Mechanisms of Eosinophil Recruitment and Degranulation

By

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STATEMENT

I, Arne W. Mould declare that all of the work presented within this thesis is my own.

a.w. Mould 9/11/98

Arne W. Mould.

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ABSTRACT

The eosinophil is a proinflammatory leukocyte that is present in the circulation and in tissues in low levels in healthy individuals. In atopics, levels of this cell are markedly elevated and eosinophilic inflammation is believed to underlie the pathophysiological changes in affected tissues. Eosinophils have been linked with the aetiology of numerous diseases, notably asthma, rhinitis and dermatitis. The targeting of eosinophil trafficking and activation presents a potential target for therapy of allergic disease. The mechanisms regulating the trafficking and activation of eosinophils are complex and are potentially modulated by numerous cytokines, chemokines, lipid mediators and low molecular weight bioactive peptides. However, the molecules and mechanisms that selectively regulate trafficking and activation of eosinophils remain unclear. Notably , the cytokines eotaxin (chemotactic cytokine) and IL-5 have been shown to have important roles in eosinophil trafficking and activation and may selectively regulate eosinophilic responses at basal states and during inflammation. Furthermore, these cytokines co-operate to regulate eosinophilia. Currently, the relationship between IL-5 and eotaxin for regulation of eosinophil trafficking and activation has not been fully elucidated.

In order to determine the roles of, and relationship between, eotaxin and IL-5 for eosinophil trafficking in the circulation and in the skin, recombinant cytokines were administered to mice intravenously and instilled into the skin, and the levels of eosinophils in each of these compartments monitored. Both IL-5 and eotaxin potently induced blood eosinophilia when administered intravenously. Moreover, they acted in co-operation. Intravenous IL-5 mobilised eosinophils from the bone marrow, while i.v. eotaxin acted independently of this compartment. IL-5 and eotaxin also potently induced tissue eosinophilia when administered to the skin and their collective actions were synergistic. IL-5 also acted in synergy with two other C-C chemokines, MIP-1α and RANTES, but not with the lipid mediators, PAF and LTB_4. These results suggest that IL-5 may selectively amplify intracellular signals activated by CCR3 ligands.

The role of eotaxin and IL-5 in the regulation of selective eosinophilia and activation in the lung was also examined by employing recombinant vaccinia viruses (rVV) engineered to express eotaxin and IL-5 *in situ*. rVV-HA-IL-5 and rVV-HA-eotaxin potently induced pulmonary eosinophilia in naive mice and co-infections at equivalent viral titres resulted in a synergistic response. However, rVV-HA-IL-5- and rVV-HA-eotaxin- induced airways eosinophilia only resulted in the induction of airways hyperreactivity and elevated levels of major basic protein in the BALF after aeroallergen provocation, and this response was CD4-dependent. Thus, IL-5 and eotaxin in conjunction with other inflammatory

mediators released during antigen processing and CD4 T-cell activation are required to promote eosinophil activation and airways hyperreactivity.

IL-5- and eotaxin- deficient mice were also employed to investigate the role of, and relationship between, each of these cytokines in eosinophilic trafficking at base line and during allergic responses. Under these conditions eosinophil levels in the bone marrow, blood and tissues were monitored in response to systemic delivery of eotaxin and IL-5 or allergen. By employing IL-5 deficient mice, this cytokine was found to be essential for the homing and migration of eosinophils into tissues in response to specific chemotactic stimuli (ie. eotaxin and IL-5) at base line. However, IL-5 was not required for eosinophil homing to allergic sites of inflammation in the skin or lung. These results suggest that other molecules are released at sites of inflammation that can take the place of IL-5 in promoting eosinophil trafficking into tissues. IL-5 was also not required for the development of blood eosinophilia in response to i.v. eotaxin. Thus, two mechanisms may exist to regulate eosinophilia in the circulation. Eotaxin regulated eosinophil recruitment to sites of allergic inflammation in the skin and controled the action of IL-5 on eosinophil differentiation in the bone marrow. Eotaxin was not required for eosinophil trafficking to the lungs during late-phase responses to aeroallergen inhalation.

The role of IL-5 and eotaxin in the development of eosinophil-mediated airways hyperreactivity was also studied by using IL-5- or eotaxin- deficient mice. Due to the close association between IL-5 and eosinophilia, the role that each factor plays individually in the development of allergic disease remains unknown. By amplifying pulmonary eosinophilia in IL-5 deficient mice by adoptive transfer of eosinophils or by i.v. administration of eotaxin, the individual roles of IL-5 and eosinophils in the development of airways hyperreactivity following aeroallergen provocation was examined. Airways hyperreactivity was induced in IL-5 deficient mice following sensitisation and aeroallergen provocation and the degree of reactivity correlated with the presence of major basic protein and the number of eosinophils in the airways. This relationship between eosinophil numbers in the airways, major basic protein levels and airways hyperreactivity was also observed in wild type mice. However, airways hyperreactivity was significantly reduced and ~~slower~~ slower in onset in IL-5 deficient mice. The i.v. adoptive transfer of donor eosinophils and the i.v. administration of eotaxin selectively amplified the accumulation of eosinophils in the airways which directly correlated with the level of airways hyperreactivity. Furthermore, although airways hyperreactivity developed in the absence of IL-5, this molecule played a significant role in amplifying the induction of airways hyperreactivity. Airways hyperreactivity was not significantly attenuated in sensitised and aeroallergen challenged eotaxin deficient mice.

In an attempt to define the elemental signals required for the trafficking of eosinophil into sites of allergic inflammation, the requirements for IL-5, IL-4, TNF-α and IL-1β in eosinophil recruitment during allergic cutaneous late-phase reactions were examined in mice using anti-cytokine mAbs and cytokine (or receptor) deficient mice. Eosinophil recruitment to sites of allergen provocation in the skin is dependent on IL-4 and TNF-α in IL-5 deficient mice, but not in wild type mice. These results suggest that IL-5 may have overlapping function with IL-4 and TNF-α and may promote eosinophil recruitment to sites of allergen provocation by activating adhesion systems at the vascular endothelium. Furthermore, eosinophil recruitment at sites of inflammation was dependent on ICAM-1 and VCAM-1 and thus, IL-4, TNF-α and IL-5 may activate these adhesion pathways.

In conclusion, IL-5 and eotaxin play fundamental roles in the regulation of eosinophil function. Notably both cytokines can regulate eosinophil differentiation, as well as blood and tissue eosinophilia. In particular, the migration of eosinophils is regulated by a unique signalling arrangement between IL-5 and chemokines that activate CCR3 which provides a mechanism that synergistically and selectively potentiates eosinophilia. Modulation of IL-5 and eotaxin signalling may provide a significant advance in the treatment of eosinophil dependent pathophysiology in allergic disease.

TABLE OF CONTENTS

CHAPTER II

THE RELATIONSHIP BETWEEN INTERLEUKIN-5 AND EOTAXIN IN THE REGULATION OF BLOOD AND CUTANEOUS EOSINOPHILIA IN MICE

CHAPTER III

THE RELATIONSHIP BETWEEN INTERLEUKIN-5 AND EOTAXIN IN REGULATING PULMONARY EOSINOPHILIA AND THEIR ROLE IN AIRWAYS DYSFUNCTION IN MICE

CHAPTER V

THE ROLE OF INTERLEUKIN-5 IN EOSINOPHIL TRAFFICKING AND FUNCTION DURING ALLERGY

CHAPTER VII

THE ROLE OF IL-5, IL-4, TNF-α and IL-1β IN EOSINOPHIL RECRUITMENT DURING ALLERGIC CUTANEOUS LATE-PHASE REACTIONS IN MICE

LIST OF FIGURES

LIST OF TABLES

Table Page

LIST OF ABREVIATIONS

BAL - bronchoalveolar lavage
BALF - bronchoaleveolar lavage fluid
BSA - bovine serum albumin
CLPR - cutaneous late-phase reaction
EC - effective concentration
ECP - eosinophil cationic protein
ECF-a - eosinophil chemotactic factor-a
EDN - eosinophil derived neurotoxin
ELISA - enzyme linked immunosorbent assay
EPO - eosinophil peroxidase
FACS - fluorescence activated cell sorter
FCS - foetal calf serum
G-CSF - granulocyte-colony stimulating factor
GM-CSF - granulocyte/macrophage-colony stimulating factor
HA - hemagglutinin
HBSS - Hank's buffered salt solution
HSV - herpes simplex virus
ICAM - intercellular adhesion molecule
Ig - immunoglobulin
IL - interleukin
IFN - interferon
i.p. - intraperitioneal
i.v. - intravenous
LB - Luria broth
LFA - lymphocyte functional antigen
LIF - leukemia inhibitory factor
LTB_4 - leukotriene B_4
mAb - monoclonal antibody
MBP - major basic protein
MCP - monocyte chemoattractant protein
MIP - macrophage inflammatory protein
M. O. I. - multiplicity of infection
M.O.P. - mini-osmotic pump
MTAGG - methotrexate
Ova - ovalbumin
PAF - platelet-activating factor
PAGE - polyacrylamide gel electrophoresis
PBS - phosphate-buffered saline

PCR - polymerase chain reaction

pfu - plaque forming unit

RANTES - regulated upon activation in normal T-cells expressed and secreted

s.c. - subcutaneous

SEM - standard error of the mean

TBS - tris-buffered saline

TK - thymidine kinase

TNF - tumour necrosis factor

VCAM - vascular cell adhesion molecule

VLA - very late antigen

-/- - deficient

+/+ wild type

LIST OF PUBLICATIONS

The following publications arose from work contained within this thesis:

Abstracts

Relationship between Interleukin-5 and Eotaxin in Regulating Blood and Tissue Eosinophilia in Mice. **Arne W. Mould**, Klaus I. Matthaei, Ian G. Young and Paul S.Foster. Postgraduate Committee in Medicine, 1996 Scientific Meeting. Canberra, ACT., Australia.

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CHAPTER I

INTRODUCTION

I.1 INTRODUCTION.

The eosinophilic leukocyte has been implicated as a primary effector cell in the pathogenesis of allergic inflammatory diseases such as asthma, rhinitis and dermatitis, in addition to inflammatory bowel disease and certain disorders of the nervous system (Gleich and Adolphson, 1986; Walsh and Gaginella, 1991). The severity and high frequency of allergic diseases in society, particularly asthma, has led to extensive investigations into the mechanisms regulating the recruitment and activation of eosinophils at sites of inflammation. To specifically target the action of the eosinophil in disease pathology, it is necessary to further elucidate the complex mechanisms that are involved in the recruitment and activation of this cell at sites of inflammation. Characterisation of these mechanisms will also aid in the further understanding of the role of eosinophils in numerous other physiological responses, such as the hosts resistance/defence against some parasites (particularly helminths), wound healing, tissue remodelling and anti-tumourogenesis (Lowe *et al.,* 1981; Todd *et al.,* 1991; Wong *et al.,* 1993; Wardlaw *et al.*, 1995).

I.2 EOSINOPHILS AND ATOPIC ALLERGIC DISEASES.

The association of eosinophils with atopic inflammatory diseases, such as asthma, rhinitis and dermatitis is well documented (Bruijnzeel *et al.*, 1989; Bousquet *et al.,* 1990; Naclerio *et al.,* 1994; Leiferman, 1994). Furthermore, there is increasing evidence that eosinophilic inflammation underlies the pathophysiological changes that are associated with these diseases (Reed, 1986; de Vries, 1994, Oddera *et al.*, 1996).

Atopic asthma is a chronic inflammatory disorder of the lung that is primarily characterised by chronic airways inflammation, reversible airways obstruction and airways hyperreactivity to inhaled spasmogens, such as β-methylcholine and histamine (Bochner *et al.,* 1994). Although the exact cause of atopic allergy (particularly asthma) remains unknown, eosinophilic inflammation is currently thought to underlie pathophysiological changes that are characteristically seen in susceptible individuals (Bousquet *et al.,* 1990; Wardlaw *et al.,* 1994; Martin *et al.,* 1996). This is particularly evident in clinical asthma and experimental animal models of asthma where there are strong correlations between eosinophilic inflammation, the induction of airways hyperreactivity to inhaled spasmogens and disease severity (Bradley *et al.,* 1991; Elwood *et al.,* 1991).

Elevated levels of activated eosinophils are present in bronchial biopsies, bronchoalveolar lavage fluid (BALF) and in the peripheral blood of patients with asthma (Bochner *et al.,* 1994). Clinical observations have also shown a correlation between eosinophil numbers

in the circulation and in the BALF of asthmatics and the severity of disease (Bousquet *et al.*, 1990; Walker *et al.*, 1991). There are, however, some clinical and experimental studies that have not found a direct correlation between the levels of eosinophils in the BALF and airways hyperreactivity (Djukanovic *et al.*, 1990; Ishida *et al.*, 1990; Banner *et al.*, 1996).

Atopic dermatitis is a chronic eosinophilic inflammatory disease of the skin which shares many similarities with asthma. Large amounts of eosinophil granular proteins have been found in biopsies of skin lesions and sufferers of this disease commonly have elevated levels of circulating eosinophils and IgE (Rajika, 1975; Leiferman *et al.*, 1985, 1990; Cheng *et al.*, 1997). Elevated levels of serum IgE are seasonal and can be related to the presence of specific environmental allergens (de Vries, 1994). Atopic individuals exhibit hypersensitivity to specific environmental allergens and usually experience a severe, often organ-specific allergic reaction within minutes of allergen exposure. Antigen-induced crosslinking of two or more IgE molecules, that are bound to mast cells and basophils via the high affinity IgE receptor ($Fc_{\gamma}II$) are thought to trigger the release of various inflammatory mediators [such as histamine and platelet activating factor (PAF)] that drive the inflammatory response.

Atopic individuals may also experience a delayed non-specific hypersensitivity reaction that occurs within 6 hours after allergen exposure and lasts up to 12 hours. In atopic dermatitis the delayed response is characterised by a cutaneous blanch reaction, while in individuals with asthma this responses takes the form of a spasmodic narrowing of the airways with an associated reduction in forced expiratory volume in one second (FEV$_1$). This hypersensitivity reaction is commonly termed the late-phase response (LPR) and is characteristically accompanied by a pronounced infiltration of inflammatory cells into the site of allergen exposure. In asthma, the late-phase response is most notably characterised by the presence of activated eosinophils and of T-lymphocytes, although other inflammatory cells such as macrophages and neutrophils may also be present. Notably, the T-lymphocytes are CD4 positive and are of a Th_2 phenotype (de Vries, 1994; Wardlaw *et al.*, 1995).

I.3 THE ROLE OF EOSINOPHILS IN PATHOGENESIS OF ALLERGIC DISEASE.

Upon activation, eosinophils have been shown to secrete a multitude of proinflammatory mediators [reactive oxygen species, lipid mediators (leukotrienes and PAF) and cationic proteins] and cytokines that have the potential to induce tissue damage and dysfunction (Bruijnzeel, 1989; Gleich, 1990, Gleich *et al.*, 1994).

Four eosinophil cationic proteins have been characterised [major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN)]. These proteins are found in the cytoplasmic granules of the eosinophil (Wardlaw *et al.*, 1995) and through a process termed degranulation, they are directionally released into the extracellular environment. Elevated levels of cationic proteins are present in the affected tissues of a number of eosinophilic diseases (Dor *et al.*, 1984; Hisamatsu *et al.*, 1995; Leonardi *et al.*, 1995). Furthermore, a correlation between the levels of these cationic proteins in the BALF and the clinical symptoms of asthma has been demonstrated (Frigas *et al.*, 1981; Wardlaw *et al.*, 1988). Some of these cationic proteins (EPO and MBP) have been shown to be potent cytotoxins for the nasal and respiratory epithelium, inducing cilliostasis and tracheal epithelial denudation, *in vitro* (Ayars *et al.*, 1989; Motojima *et al.*, 1989; Tagari *et al.*, 1992). In studies involving primates, the direct instillation of recombinant human MBP to the lung was able to enhance airway hyperresponsiveness to β-methylcholine. Moreover, this effect of MBP was dose dependent and required an intact epithelium (Flavahan *et al.*, 1988; Gundel *et al.*, 1991). Cytotoxic concentrations of MBP are found in airways of asthmatics and at sites of inflammation in other allergic diseases (Gleich *et al.*, 1979). Studies in rats have also show a role for MBP in the induction of epithelial-dependent airway hyperreactivity (Coyle *et al.*, 1994). MBP-induced airways hyperreactivity in rats may be dependent on the generation of i-kinins, such as bradykinin (Coyle *et al.*, 1995). i=immunoreactive.

However, MBP and other cationic proteins may induced airways hyperreactivity by multiple mechanisms. MBP and EPO may enhance vagally mediated bronchoconstriction by acting as allosteric inhibitors of muscarinic M2 receptors (Gleich *et al.*, 1995), in additon MBP may also activate platelets, neutrophils, mast cells and basophils to release numerous proinflammatory cytokines and lipid mediators (O'Donnel *et al.*, 1983; Rohrbach *et al.*, 1990; Moy *et al.*, 1990).

I.4 MECHANISM OF EOSINOPHIL TRAFFICKING AND DEGRANULATION.

Clinical observations and investigations in animal models of allergy are slowly elucidating the complex mechanisms involved in the trafficking of eosinophils in inflammatory diseases. During an allergic response eosinophils must traffic from the bone marrow compartment to the site of inflammation. Eosinophil migration and activation are regulated by 6 key events: 1. Inflammatory stimuli at the site of inflammation initiate a series of signalling events that leads to the differentiation of a committed eosinophil/basophil precursor cell within the bone marrow (or in the circulation) into mature eosinophils; 2. Eosinophils residing in the bone marrow enter

the blood stream; 3. Inflammatory mediators (specific cytokines and lipid mediators) released at the sites of inflammation upregulate the expression of specific adhesion molecules on the vascular endothelium and on the eosinophil and prime this cell for chemotactic and degranulatory responses; 4. At sites of inflammation, activated eosinophils bind to complementary adhesion molecules on the vascular endothelium and undergo diapedesis; 5. Eosinophils directionally migrate towards the site of inflammation (chemotaxis) in response to chemotactic signals elicited by cytokines, chemokines and lipid mediators that are produced at the site of inflammation. In addition, locally produced cytokines prolong eosinophil survival by inhibiting apoptosis; 6. Signalling events involving specific inflammatory mediators, such as allergen-specific immunoglobulins, activate eosinophils resulting in the release of oxygen radicals, lipid mediators and cytotoxic cationic proteins which promote tissue dysfunction and disease pathology.

I.4.1 Eosinophil differentiation.

As with all cells of the lymphomyeolid lineage, eosinophils are derived from haemopoietic stem cells which reside in the bone marrow (Wardlaw *et al.,* 1995). In a complex mechanism of interaction, potentially involving interleukin (IL) -6, IL-11, IL-12, granulocyte-colony stimulating factor (G-CSF), leukemia-inhibitory factor (LIF) and steel factor, haemopoietic stem cells are committed into multipotential $CD34^+$ progenitors (Ogawa, 1994).

In the presence of IL-3 and/or granulocyte macrophage-colony stimulating factor (GM-CSF), $CD34^+$ myeloid progenitor cells undergo committed differentiation into $CD34^+$ basophil/eosinophil myeloid progenitor cells. The intravenous administration of IL-3 and GM-CSF synergistically stimulates an increase in eosinophil/basophil precursor levels in the circulation (Donahue *et al.,* 1988). However, the involvement of IL-3 and/or GM-CSF in the generation of eosinophil precursor cells is not mandatory, as mature eosinophils are found in mice with impaired IL-3 and GM-CSF function (Nishinakamura *et al.,* 1996). Although eosinophil/basophil progenitor cells are predominantly found in the bone marrow, these cells are also present in the circulation, particularly in atopic individuals (Denburg *et al.,* 1983, 1985a, 1985b).

In the presence of either IL-3, IL-5 or GM-CSF individual basophil/eosinophil precursors cells may undergo differentiation/proliferation to form homozygous colonies of either the basophil or eosinophil lineage or ~~heterozygous~~ mixed basophil/eosinophil colonies. Generally, IL-3 and IL-5 preferentially direct and stimulate the differentiation of human basophil/eosinophil precursor cells towards the basophil and eosinophil lineages, respectively (Sanderson *et al.,* 1985; Meyer *et al.,* 1989; Valent *et al.,* 1989). In

contrast, GM-CSF does not appear to be selective in its action on the differentiation of basophil/eosinophil progenitor cells and gives rise to mixed colonies when added to peripheral blood-derived basophil/eosinophil precursor cells *in vitro* (Denburg *et al.*, 1991).

In the murine system, IL-5 is a potent inducer of basophil/eosinophil progenitor differentiation towards the eosinophil lineage in culture, giving rise to almost pure eosinophil colonies (Sanderson *et al.*, 1985). In contrast, IL-3 appears to act primarily as a mast cell growth factor (Ihle *et al.*, 1983). Both IL-3 and GM-CSF are regarded as multi-haemopoietins and are potentially involved in the committed-differentiation of many different myeloid lineages (Metcalfe, 1993). IL-5 on the other hand appears to be primarily an eosinophil differentiation factor (Denburg *et al.*, 1994). Notably, IL-5 also plays a key role in the later stages of eosinophil differentiation. However, mice genetically altered for the over expression of this cytokine exhibit pronounced blood and tissue eosinophilia (Clutterbuck *et al.*, 1989; Dent *et al.*, 1990; Tominaga *et al.*, 1991; Shalit *et al.*, 1995). This indicates that IL-5 may also directly or indirectly (via the stimulation of other signalling molecules) influence early myeloid progenitor commitment towards the eosinophil lineage. In contrast, mice expressing high levels of IL-3 or GM-CSF exhibit only mild blood eosinophilia and have high levels of cells of other myeloid lineages, such as neutrophils and macrophages (Chang *et al.*, 1989; Johnson *et al.*, 1989). Interestingly, mice genetically altered for functional loss of IL-5, IL-3 and GM-CSF possess morphologically mature eosinophils, albeit at lower levels (Nishinakamura *et al.*, 1996). This suggests there is a degree of redundancy in the action of these three cytokines in basal eosinophil development. IL-5, IL-3 and GM-CSF may have a more important role in enhancing eosinophil production during inflammation where levels of each of these cytokines are elevated (Walker *et al.*, 1992).

Studies by Perez *et al.*, (1993) supports the existence of an IL-5, IL-3 and GM-CSF independent pathway for eosinophil proliferation. The cell-free pleural washings from rats given an injection of platelet-activating factor (PAF) into the pleural cavity stimulated murine haemopoietic eosinophil proliferation *in vitro* and this response was not inhibited by neutralising antibodies to IL-5, IL-3 or GM-CSF given alone or in combination to the cell culture. The role of cytokines in eosinophil differentiation is summarised in figure I.1.

I.4.2 Eosinophil Priming.

Circulating eosinophils in atopic individuals are morphologically and functionally dissimilar to those in the circulation of non-atopics (Kermal *et al.*, 1990; Griffin *et al.*, 1991). Eosinophils from atopic individuals are characteristically hypodense and may be

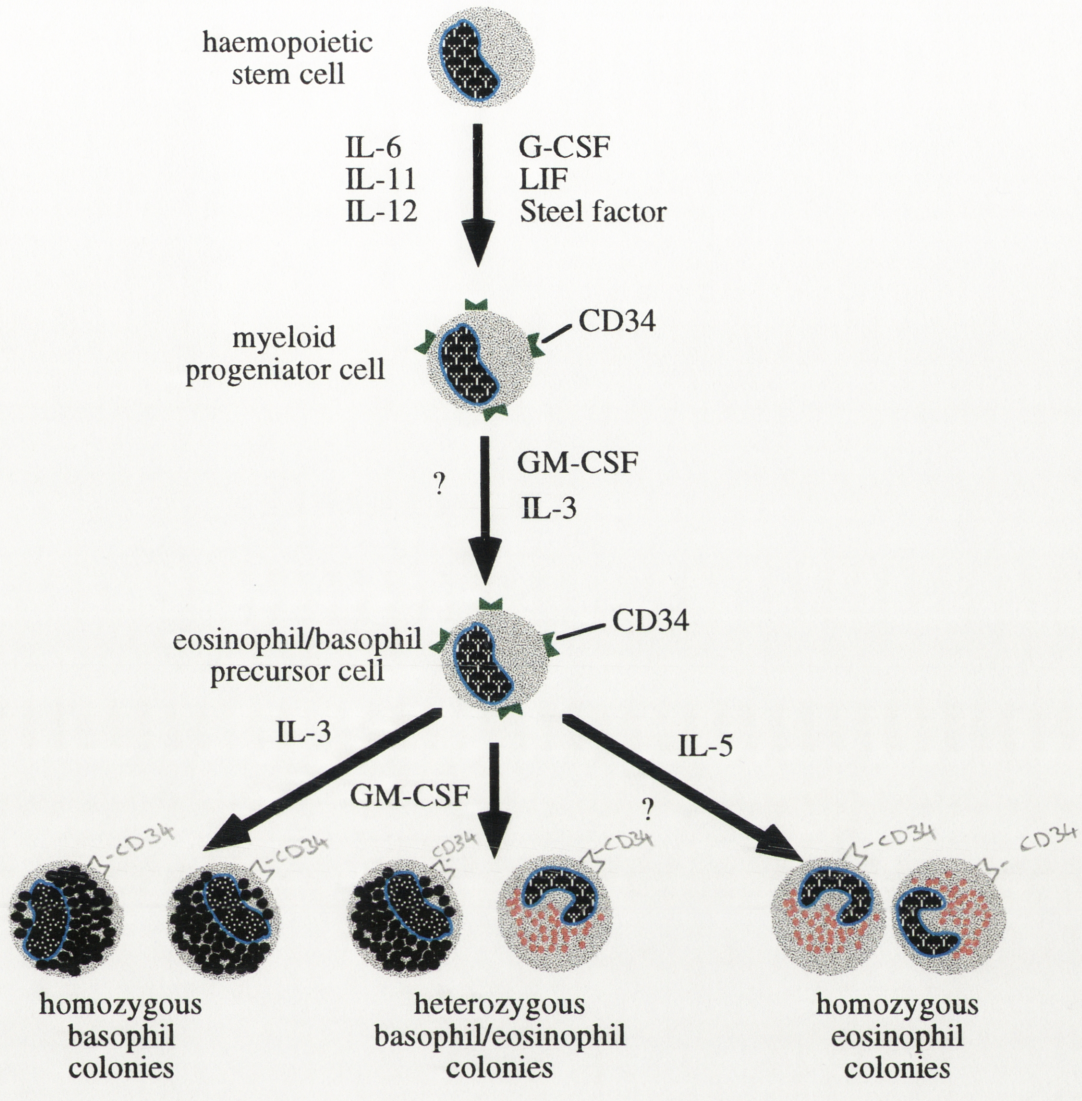


Figure I.1 ***Eosinophil differentiation.***

Haemopoietic stem cells undergo differentiation into CD34+ myeloid progenitor cells in a complex interaction that is thought to involve IL-6, IL-11, IL-12, G-CSF, LIF and Steel factor. In the presence of GM-CSF and IL-3 this cell population undergoes committed differentiation into an eosinophil/basophil progenitor cell. Eosinophil/basophil progenitor cells differentiate toward eosinophil or basophil lineages and this process is probably regulated by IL-5, IL-3 and GM-CSF (Wardlaw et al., 1995).

separated from those of a healthy individual by Percoll density gradients (Fukuda *et al.*, 1985). Associated with this change in eosinophil density is the enhancement of numerous eosinophil functional responses, including mobility (chemotaxis), cellular adhesion and terminal effector function (ie. degranulation) (Carlson *et al.*, 1991; Warringa *et al.*, 1992a; Li *et al.*, 1996). The enhanced chemotactic responses of eosinophils isolated from atopic individuals to PAF is well documented with cells showing strong chemotactic responses at concentrations of this molecule that are suboptimal for eosinophils from a healthy donor (Morita, *et al.*, 1989; Warringa *et al.*, 1992a). Hypodense eosinophils isolated from the blood of atopics also exhibit strong chemotactic responses to IL-8, IL-4 and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Eda *et al.*, 1993; Warringa *et al.*, 1993; Dubois *et al.*, 1994). In contrast, eosinophils from normal donors respond only weakly or not at all to these molecules. The hypodense phenotype is thought to be effected by mediators of inflammation such as PAF and the cytokines, IL-3, GM-CSF and IL-5 (Yukawa *et al.*, 1989; Koenderman *et al.*, 1996). Low density eosinophils are also found in individuals afflicted with the hypereosinophilic syndrome (HES), however, although eosinophil hypodensity has been associated with eosinophil priming, this lower density phenotype does not always confer enhanced chemotactic responsiveness to PAF (Little and Casale, 1991).

I.4.2.1 *The enhancement of eosinophil chemotaxis and migration by priming.*

Allergic inflammation in thought to underlie disease pathology in atopics and is primarily characterised by elevated circulating levels and tissue infiltration of activated eosinophils and $CD4^+$ T-lymphocytes. This population of T-lymphocytes secrete cytokines of a Th_2 type, including IL-4, IL-5 and IL-10. Th_2 cells also produce GM-CSF and IL-3. Although weak chemotactic agents for non-primed human eosinophils *in vitro*, IL-5, IL-3 and GM-CSF may have a major role in the priming of eosinophil function whilst in the circulation (Semhi *et al.*, 1992). IL-5 has been shown to selectively enhance the *in vitro* chemotactic responses of normal human eosinophils, but not neutrophils, to lipid mediators [PAF, leukotriene B_4 (LTB_4)], fMLP and chemokines of both C-C [Regulated upon activation in normal T-cells expressed and secreted (RANTES)] and C-X-C (IL-8) families, as measured *in vitro* with Boyden chambers (Sehmi *et al.*, 1992; Schweizer *et al.*, 1994).

The ability of IL-5 to enhance the chemotactic responses of eosinophils to molecules, such as PAF, may involve the upregulation of chemoattractant receptors on the eosinophil (Kishimoto *et al.*, 1996). The preincubation of normal human eosinophils with pico-molar concentrations of GM-CSF or IL-3 enhances the *in vitro* chemotactic response of these cells to LTB_4 and PAF, respectively. Individually, both of these cytokines

stimulate normal eosinophils to chemotactically respond to IL-8 and fMLP (Warringa *et al.*, 1991). A role for IL-5 in the priming of eosinophils *in vivo* has also been shown. Eosinophils purified from allergic guinea pigs are more responsive to chemotactic stimuli (including PAF and LTB_4) *in vitro*, than non-allergic controls (Coeffeir *et al.*, 1994). Furthermore, the pretreatment of these allergic animals with anti-IL-5 mAb, before antigen challenge reduces the *in vitro* chemotactic responsiveness of eosinophils to PAF and LTB_4.

In addition to enhancing chemotaxis, cytokines may prime eosinophils for enhanced adhesion to the vascular endothelium through the activation of adhesion systems. GM-CSF activates the functional adhesive state of vascular cell adhesion molecule-4 (VLA-4) on eosinophils without any apparent upregulation of expression (Paul-Sung *et al.*, 1997). Both PAF and platelet factor-4 increase the adhesion of eosinophils to tissue culture plates coated with soluble intercellular adhesion molecule-1 (ICAM-1) and this process may involve the induction of the expression of lymphocyte function-associated antigen-1α (LFA-1α) and LFA-1β on the eosinophil (Hayashi *et al.*, 1994). Certain neuropeptides (substance P, neurokinin A, calcitonin gene-related peptide and cholecystokinin octapeptide) also have priming effects leading to enhanced chemotactic responsiveness of eosinophils to PAF and LTB_4 *in vitro*. Interestingly, the priming ability of these neuropeptides was reported to only be active on eosinophils isolated from the blood of atopics (Nuamo and Agrawal, 1992).

I.4.2.2 *Eosinophil cytotoxicity and cytokine priming.*

The priming of eosinophils by cytokines does not only lead to enhanced chemotactic/migrational responses. In the presence of some cytokines eosinophils display hypersensitivity to degranulatory stimuli. IL-5, IL-3 and GM-CSF all enhance immmunoglobulin-dependent eosinophil degranulation *in vitro*, while IL-1, IL-2, IL-4, IL-6 and TNF-α appear to have no effect (Fugisawa *et al.*, 1990). One cytotoxic property of eosinophils involves the generation and release of reactive oxygen species through a process termed respiratory burst. The stimulation of respiratory bursts in human eosinophils, induced by serum-treated zymosan particles, is also enhanced by the prior exposure of the cells to either IL-5, IL-3 or GM-CSF (Van de Bruggen *et al.*, 1993; Koenderman *et al.*, 1996).

I.4.3 The role of adhesion molecules in eosinophil trafficking.

The preferential accumulation of eosinophils, but not neutrophils, at sites of inflammation is a hallmark of many atopic inflammatory diseases. For eosinophils to migrate from the circulation into inflamed tissues in response to chemotactic signals they must cross the

vascular endothelium. In order to do this eosinophils utilise a number of adhesion systems that are activated by inflammatory cytokines and lipid mediators. Eosinophil transendothelial migration is thought to regulated by the selectin and integrin/immunoglobulin families in concert with unidirectional chemotactic gradients generated at the site of inflammation (reviewed by Hogan and Foster, 1996).

I.4.3.1 *The role of selectins in eosinophils adhesion to the vascular endothelium.*

Selectins are a family of fucosylated carbohydrate ligand-binding adhesion molecules that include L-, P- and E- subtypes (named according to their expression on leukocytes, platelets and endothelial cells), all of whom share a common N-terminal lectin and epithelial growth factor like domains (Wardlaw *et al.,* 1995).

L-selectin is expressed on all circulating leukocytes including eosinophils, while both E- and P-selectins are expressed primarily on endothelial cells following cytokine activation (Bevilacqua *et al.,* 1987; Tedder *et al.,* 1995). The initial tethering of eosinophils to, and their subsequent rolling along, the vascular endothelium during early events of transendothelial migration has not been fully characterised, but may involve adhesion processes that utilise L-selectin and P-selectin (Wardlaw *et al.,* 1994). Antibodies against L-selectin block eosinophil adhesion to cytokine activated endothelial cells *in vitro* (Knol *et al.,* 1994). Interestingly, although neutrophils reportedly use L-selectin to tether to the activated endothelium, the adhesion of neutrophils and eosinophils to the vascular endothelium through this molecule has been shown to involve different mechanisms (Knol *et al.,* 1994). This suggests that the binding of L-selectin to its ligand(s) at the vascular endothelium is cell specific.

A role for L-selectin in eosinophil tethering and rolling along the vascular endothelium has been demonstrated in rabbit mesenteric venules *in vivo* (Sriramarao *et al.*, 1994). Interestingly, PAF, IL-5 and IL-3 all induce the shedding of L-selectin from eosinophils *in vitro* (Smith *et al.,* 1992; Needley *et al.,* 1993). The shedding of L-selectin by these cytokines co-incides with the upregulation of another adhesion molecule, Mac-1, on the surface of the eosinophil. Mac-1 may enhance anchoring and transmigration of the eosinophil through activated endothelium by interaction with the counter ligand, ICAM-1 (Zimmerman *et al.,* 1992; Ebisawa *et al.,* 1992). It has also been suggested that the shedding of L-selectin and the subsequent upregulation of Mac-1 on the eosinophil is probably induced by physical interactions between this leukocyte and the endothelium (Ebisawa *et al.*, 1992). Eosinophils isolated from the BALF of asthmatic patients following aeroallergen provocation have decreased levels of membrane associated

L-selectin in comparison to eosinophils isolated from the blood of the same individuals (Georas *et al.,* 1992) and thus, the shedding of L-selectin may be necessary for transmigration to occur (Wardlaw *et al.,* 1994).

The role of E-selectin and P-selectin in eosinophil binding to the vascular endothelium is not clear. Tissue culture plates coated with recombinant P- or E- selectins bind eosinophils and adherence is inhibited by the addition of antibodies raised against the respective selectin (Weller *et al.,* 1991; Wein *et al.,* 1995). Other *in vitro* studies, however, do not support a physiological role for E-selectin in the adherence of eosinophils to the activated endothelium (Sriramarao *et al.,* 1996). Notably, E-selectin only weakly binds eosinophils under conditions of shear force which mimic the dynamics of blood flow. Conversely, there is strong evidence to suggest that P-selectin is involved in the recruitment of eosinophils to nasal polyps, possibly by promoting the initial tethering of eosinophils to the vascular endothelium at this site (Symon *et al.,* 1994; Wardlaw *et al.,* 1994).

I.4.3.2 *The role of integrins and immunoglobulin family adhesion molecules in eosinophil transmigration.*

Following the initial tethering and rolling of leukocytes along the vascular endothelium, stronger cell adhesion needs to take place for transmigration to occur (Zimmerman *et al.,* 1992). Both the integrin and the immunoglobulin families of adhesion molecules are thought to participate in this process of firm adherence of eosinophils to the endothelium (reviewed in Wardlaw *et al.,* 1995).

I.4.3.2.1 *The integrins.*

The integrins are a superfamily of α/β heterodimeric transmembrane glycoproteins that function as adhesion molecules in cell-cell and cell-substratum interactions. Adhesion molecules from this superfamily are predominantly expressed on the surface of leukocytes and bind to counter ligands that are expressed on the surface of endothelial cells and other leukocytes (Haas and Plow, 1994; Hoire *et al.,* 1997). Eosinophils express the β_2 integrins, LFA-1(CD11a/CD18) and Mac-1 (CD11b/CD18) and the $\alpha_4\beta_1$ integrin, VLA-4. Importantly, VLA-4 is expressed on eosinophils, but not on neutrophils, suggesting a key role for this molecule in the selective migration of eosinophils into inflamed tissues in atopic disease (Nourshargh, 1993).

There are two known ligands for VLA-4, fibronectin and VCAM-1 (Elices *et al.,* 1990) This suggests that VLA-4 may be involved in eosinophil adhesion to the vascular endothelium as well as migration within tissues. VLA-4 and L-selectin both mediate the

rolling of eosinophils along venules (Sriramarao *et al.,* 1994). In contrast, neutrophils are thought to predominantly use L-selectin in this phase of the migrational process. Eosinophils isolated from the blood of allergic asthmatics, but not those isolated from normal individuals, spontaneously transmigrate through IL-1β- or TNF-α- stimulated monolayers of human umbilical vascular endothelial cells (HUVEC). Transmigration may reflect cytokine activation of eosinophils in asthmatics, since normal eosinophils primed with either IL-5, IL-3 or GM-CSF also undergo spontaneous transendothelial migration in this *in vitro* system. Thus, transendothelial migration not only requires activation of the endothelium, but also of the eosinophil.

Studies using IL-4-stimulated HUVEC also showed that eosinophils isolated from individuals with atopic dermatitis spontaneously transmigrate through monolayers in comparison to eosinophils isolated from normal donors (Moser *et al.,* 1992b). Furthermore, in contrast to IL-1β or TNF-α, the stimulation of HUVEC with IL-4 does not promote spontaneous neutrophil transmigration. Eosinophil transmigration through IL-4-activated monolayers of HUVEC was inhibited by anti-CD11/CD18 and anti-VLA-4 mAbs (Moser *et al.,* 1992b). Although GM-CSF is known to control the adhesive properties of VLA-4 on the eosinophil, transmigration through IL-1β activated monolayers of HUVEC was inhibited only by anti-CD11/CD18 mAb, but not anti-VLA-4 mAb (Moser *et al.,* 1992a; Paul Sung *et al.,* 1997). This indicates that eosinophil transmigration can occur independently of VLA-4 and that in different cytokine environments eosinophils may use different adhesion pathways during transendothelial migration. IL-1β appears to promote transmigration via the CD11/CD18 adhesion pathways while IL-4 promotes transmigration via both CD11/CD18 and VLA-4 adhesion pathways. These studies also indicated the complementary adhesion molecules for both CD18/CD11 and VLA-4 are present on the atopic eosinophil. The observation that IL-4 may activate VLA-4 on the eosinophil, but not on the neutrophil, implies that this cytokine plays a role in the selective trafficking of eosinophils during allergic inflammation (Moser, 1994).

A role for VLA-4 in the mechanism of eosinophil accumulation *in vivo* has also been demonstrated. Pretreatment of guinea pigs with anti-VLA-4 mAb was shown to inhibit the accumulation of eosinophils in the skin is response to LTB_4 or PAF and during passive cutaneous anaphylaxis, as well as in the nasal mucosa following antigen inhalation (Weg *et al.,* 1993; Terada *et al.,* 1996).

I.4.3.2.2 *Immunoglobulin superfamily.*

ICAM-1 and VCAM-1 are two members of the immunoglobulin superfamily of adhesion molecules that regulate eosinophil adhesion *in vivo*. ICAM-1 is weakly expressed on the

endothelium at non-inflamed sites and its expression is upregulated on endothelial cells by inflammatory cytokines (IFNγ, IL-1β and TNF-α) and lipopolysacharide (LPS) (Acevedo *et al.,* 1993). ICAM-1 acts as a ligand for both LFA-1 and Mac-1 and this interaction is potentially involved in the trafficking of numerous type of leukocytes (eosinophils, neutrophil and lymphocytes) (Marlin and Springer, 1987). ICAM-1 is involved in the adhesion of eosinophils to IL-1 (isotype undefined)-, TNF-α- or LPS-stimulated endothelial cultures by a mechanism involving CD18-dependent interactions (Lamas *et al.,* 1988).

In contrast to ICAM-1, VCAM-1 is not detectable on resting endothelial cells. However, as with ICAM-1 the expression of VCAM-1 is upregulated in the presence of IFNγ, IL-1β and TNF-α. IL-4 also stimulates the expression of VCAM-1 on cultured endothelial cells, although this cytokine has little effect on ICAM-1 expression (Shimizu *et al.,* 1992). VCAM-1 binds the VLA-4 which is expressed on eosinophils but not neutrophils. The selective expression of this molecule on eosinophils suggests that the VCAM-1/VLA-4 pathway plays a prominent role in regulating eosinophilic inflammation in allergic disease (Nakajima *et al.,* 1994; Jahnsen, 1995; Tanimukai *et al.,* 1995).

The expression of ICAM-1 on the vascular endothelium is upregulated in inflamed tissues of allergic individuals following allergen provocation (Kyan-Aung *et al.,* 1991; Montefort *et al.,* 1994). Increased ICAM-1 expression in the pulmonary vasculature of asthmatic patients has also been reported, although only in patients suffering from intrinsic and not extrinsic disease (Bentley *et al.,* 1993, 1994). VCAM-1 expression on the vascular endothelium is also elevated in numerous allergic diseases and often correlates with the number of infiltrating eosinophils (Jahnsen *et al.,* 1995; Nakumura, 1996).

A role for VCAM-1/VLA-4 interactions, but not the ICAM-1/LFA-1 adhesion system, in allergen-induced accumulation of eosinophils in the lungs of mice has been demonstrated by using blocking antibodies to each of these adhesion pathways (Nakajima *et al.,* 1994). Furthermore, the inhibition of VCAM-1 and VLA-4 function, but not that of ICAM-1 or LFA-1 (with inhibitory mAbs) caused an increase in blood eosinophil numbers, suggesting a role for VCAM-1 and VLA-4 in the adhesion of eosinophils to the vascular endothelium in inflamed tissues. However, other studies using a sephadex-induced pulmonary eosinophilia model in guinea pigs demonstrated that the accumulation of eosinophils in the BALF was dependent on both VLA-4 and CD18-mediated pathways (Das *et al.*, 1995).

In summary, current literature suggests that eosinophils may potentially use both the ICAM-1/CD11/CD18 and the VCAM-1/VLA-4 cellular adhesion pathways in the process of transendothelial migration, however, the specific expression of VLA-4 on eosinophils

indicates that adhesion systems utilising this molecule selectively regulate the migration of this leukocyte into inflamed tissues during allergic responses.

The role of adhesion molecules in eosinophil adhesion and transendothelial migration is summarised in figure I.2.

I.4.4 Molecules regulating eosinophil trafficking in tissues.

The accumulation of eosinophils in specific tissues is a prominent feature of atopic asthma and dermatitis. The mechanism by which eosinophils directionally migrate through tissues is thought to be mediated by the local release of chemoattracts and the generation of chemotactic gradients. For many years researchers have been trying to identify the molecule(s) involved in the selective accumulation of eosinophils at sites of inflammation. Numerous molecules (lipid mediators, small molecular weight peptides, cytokines and chemokines) induce eosinophil chemotaxis *in vitro* and/or regulate trafficking of this leukocyte *in vivo*. However, the majority of these molecules also act as chemoattractants for other leukocytes, questioning their relative roles and importance in eosinophil trafficking in many disease states (Wardlaw *et al.*, 1995).

I.4.4.1 *Small molecular weight peptides and eosinophil trafficking.*

Numerous small molecular weight peptides, including fMLP, the eosinophil chemotactic factor-A (ECF-A) tetra-peptides (Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu) and histamine, are all chemotactic for eosinophils *in vitro* (Clark *et al.*, 1975; Goetzl and Austen, 1976; Ogawa *et al.*, 1981). Extensive research in the 1970s and early 1980s into the role of histamine and ECF-A in eosinophil function was undertaken as these molecules were considered to be prime candidates for the regulation eosinophil trafficking in eosinophilic disorders. Histamine and ECF-A were shown to selectively induce the chemotaxis of eosinophils *in vitro* (Clark *et al.*, 1975; Turnbull *et al.*, 1977). Furthermore, the administration of ECF-A to primates promoted the recruitment of eosinophils to sites of instillation (Turnbull *et al.*, 1977). Enthusiasm for these molecules as key regulators of eosinophil trafficking *in vivo* was reduced, however, after Wardlaw *et al.*, (1986) showed that both of these molecules were relatively weak *in vitro* chemoattractants for human eosinophils in comparison to PAF.

C5a, a peptide derivative of complement, is also a potent eosinophil chemoattractant *in vitro* and promoted the accumulation of eosinophils in the skin of guinea pigs (Kay *et al.*, 1973; Faccioli *et al.*, 1991; Pettipher *et al.*, 1994). However, C5a may regulate the accumulation of eosinophils in guinea-pig skin through the generation of other eosinophil

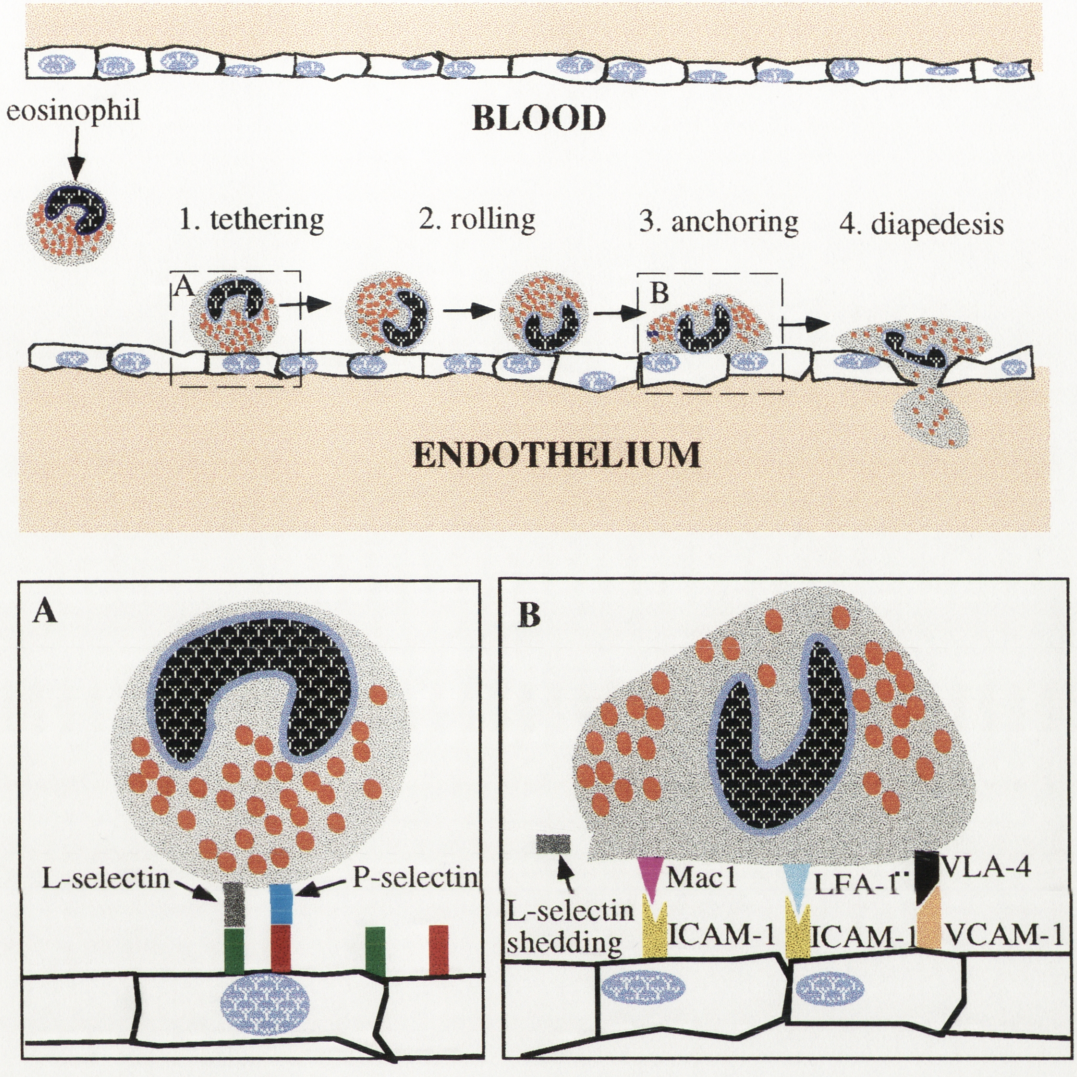


Figure I.2 ***The role of adhesion molecules in eosinophil trafficking.***

Transendothelial migration of eosinophils involves the tethering, rolling and anchoring of the eosinophils to the vascular endothelium, followed by diapedesis. Tethering (A) may be regulated by L- and P-selectin while (B) firm anchoring of the eosinophil to the vascular endothelium appears to involve Mac-1/ICAM-1, LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions. Firm anchoring of eosinophils is associated with the shedding of L-selectin and precedes the active process of diapedesis.

chemoattractants such as LTB_4 (Pettipher *et al.*, 1994). C5a is also a potent chemoattractant for neutrophils *in vitro* and *in vivo* (Fischer and Czarnetzki, 1982; Collins *et al.*, 1993; Teixeira *et al.*, 1994).

I.4.4.2 *The role of lipid mediators in eosinophil trafficking.*

The lipid mediators, PAF and LTB_4, potently induce eosinophil chemotaxis *in vitro* and regulate the accumulation of this leukocyte at sites of administration *in vivo* (Czarnetzki and Mertensmeier, 1985; Bisgaard *et al.*, 1986; Sun *et al.*, 1991; e Sliva *et al.*, 1991). However, these two lipids are not selectively chemotactic for eosinophils, acting *in vitro* as potent chemoattractants for a range of leukocytes, particularly neutrophils (Czarnetzki and Mertensmeier, 1985; Bruijnzeel *et al.*, 1990).

1.4.4.2.1 *Platelet-activating factor.*

PAF, also known as PAF-acether, is a potent chemotactic agent for human eosinophils *in vitro* (Sigal *et al.*, 1987; Tamura *et al.*, 1987; Sun *et al.*, 1991). Eosinophils isolated from the blood of atopics exhibit stronger chemotactic responses to PAF than eosinophils that are isolated from the blood of non-allergic individuals (Warringa *et al.*, 1992a). The enhanced responsiveness of eosinophils from atopic individuals is thought to be related to a higher cell activation (priming) state (Warringa *et al.*, 1992b). In support of this theory is the ability of IL-5, IL-3 and GM-CSF (all elevated at sites of allergic disease) to prime normal eosinophils for enhanced chemotactic responsiveness to PAF *in vitro* (Warringa *et al.*, 1992b). IL-5 may enhance chemotactic responses to PAF by inducing the expression of the PAF receptor (Kishimoto *et al.*, 1996).

PAF also induces the transmigration of eosinophils through monolayers of HUVEC *in vitro* (Sano *et al.*, 1995). This response is enhanced by the activation of HUVEC with either IL-1β or IL-4, indicating that PAF may act in synergy with inflammatory cytokines to promote the accumulation of eosinophils *in vivo*. PAF also induces transmigration of neutrophils across IL-1β-stimulated HUVEC, though interestingly, not across IL-4 stimulated cultures (Sano *et al.*, 1995). This indicates that PAF may act in concert with IL-4 to promote the selective transendothelial migration of eosinophils *in vitro* and collectively both molecules may play an important role in the selective accumulation of eosinophils (but not neutrophils) into inflamed tissues.

The administration of PAF induces a localised eosinophilia in both primates and rodents (Arnoux *et al.*, 1988; Coyle *et al.*, 1988). In man, the injection of PAF induces eosinophilia at the site of administration in atopics, but not in non-atopic individuals (Henocq and Vargaftig, 1986, 1988; Fadel *et al.*, 1990). Similar observations have also

been reported in mice where the intradermal administration of PAF leads to a localised eosinophilia in antigen-sensitised, but not in non-sensitised animals (Yukawa *et al.*, 1992). This indicates that eosinophils become responsive to PAF only after priming with inflammatory mediators in mice and humans. In contrast, the inhalation of PAF by naive guinea pigs and primates leads to pulmonary eosinophilia (Lellouch-Tubiana *et al.*, 1987; Coyle *et al.*, 1988; Arnoux *et al.*, 1988; Sanjar *et al.*, 1990; Wegner *et al.*, 1992). While numerous reports indicate that PAF is a potent inducer of the accumulation of eosinophils *in vivo*, eosinophil accumulatory responses to the administration of this molecule are often delayed, peaking at 24-48 hours later and may involve the generation of other eosinophil chemoattractants (Sanjar *et al.*, 1990; e Silva *et al.*, 1991). Furthermore, PAF also induces the accumulation of mononuclear cells and neutrophils at sites of administration *in vivo* (e Silva *et al.*, 1991).

Although a role for PAF in eosinophil trafficking during allergy has been demonstrated in various rodent models of allergic inflammation (Coyle *et al.*, 1988; Etienne *et al.*, 1989; Iwamoto *et al.*, 1992), other studies have failed to show any significant role for PAF in eosinophil recruitment *in vivo* (Richards *et al.*, 1991a; Johnson *et al.*, 1992).

I.4.4.2.2 *LTB$_4$*.

Although some studies have shown LTB_4 to be a potent eosinophil chemoattractant *in vitro* others have indicated that its action is relatively weak (Uden *et al.*, 1983; Bruijnzeel *et al.*, 1993). These observations may be dependent not only on the species from which the eosinophils are isolated, but also on the activational state of the cells being assayed (Sun *et al.*, 1991). LTB_4 is a weak *in vitro* chemoattractant for eosinophils isolated from normal human blood or the peritoneal cavity of monkeys (Sun *et al.*, 1991). In contrast, eosinophils isolated from the peritoneal cavity of guinea pigs exhibit strong chemotactic responses to LTB_4. Interestingly, the converse is true for PAF (Sun *et al.*, 1991). Although LTB_4 is chemotactic for both eosinophils and neutrophils *in vitro*, this molecule is preferentially chemotactic for eosinophils when compared on a molar basis. Studies in guinea pigs, however, show the converse *in vivo*, with large numbers of neutrophils accumulating at skin sites of LTB_4 administration (Czarnetzki and Mertensmeier, 1985). Unlike PAF, rather than enhanced chemotaxis to LTB_4, eosinophils obtained from atopic patients are less responsive to this lipid mediator than are cells isolated from non-atopic individuals (Czarnetzki and Rosenbach, 1986).

IL-5, IL-3 and GM-CSF all have the ability to prime eosinophils that are isolated from normal donors, but not from atopics, for enhanced chemotactic responses to LTB_4 (Sehmi *et al.*, 1992). Notably, pico-molar concentrations of GM-CSF primed eosinophils for enhanced chemotactic responses to LTB_4 *in vitro*, while nano-molar

concentrations of this cytokine had the opposite effect (Waringa *et al.*, 1991). Thus, the reduced chemotactic responsiveness of eosinophils from atopic individuals may be due to the exposure of these cells to elevated levels of GM-CSF.

Elevated levels of LTB_4 and 20-hydroxy-LTB_4 are found in the BALF of asthmatics (Lams *et al.*, 1988; Wardlaw *et al.*, 1989). Furthermore, eosinophils isolated from the BALF of atopic patients following segmental antigen challenge have a reduced chemotactic response to LTB_4 (but not fMLP) in comparison with eosinophils isolated from the blood of the same individuals (Kim *et al.*, 1994). These observations suggest that previous exposure of BALF eosinophils to LTB_4 results in desensitisation to this molecule (Kim *et al.*, 1994).

Similar observations *in vivo* have also been reported. 20-hydroxy-LTB_4 induced a significant accumulation of eosinophils at skin sites of administration in non-atopic asthmatics, but not in atopic asthmatics or normal individuals. This may indicate a role for LTB_4 and 20-hydroxy-LTB_4 in eosinophil trafficking in non-atopic asthma (Bruijnzeel *et al.*, 1993). The exposure of rats to an aerosol of LTB_4 induced a delayed pulmonary eosinophilia similar to that observed in actively sensitised rats following antigen inhalation (Richards *et al.*, 1991a). Antigen-induced BALF eosinophilia in actively sensitised guinea pigs and rats was also inhibited by an LTB_4 antagonist (Richards *et al.*, 1991a, 1991b). Similarly, the treatment of sensitised mice with an inhibitor of LTB_4 synthesis also inhibited antigen-induced BALF eosinophilia (Henderson *et al.*, 1996). Interestingly, LTB_4 antagonists had no effect on the antigen-induced influx of eosinophils into the trachea of actively sensitised dogs (Johnson *et al.*, 1992). Thus, although this molecule appears to play a significant role in the accumulation of eosinophils at sites of allergic inflammation in rodents, it may be of less importance in eosinophil recruitment during allergic responses in other species.

I.4.4.3 *The role of cytokines in eosinophil trafficking.*

Accumulating evidence indicates an important role for cytokines, particularly members of a subfamily of cytokines known as chemokines, in the mechanisms of eosinophil trafficking *in vivo* (reviewed by Hogan and Foster, 1996). Numerous cytokines (IL-1β, IL-2, IL-4, IL-5, GM-CSF and TNF-α) and chemokines [eotaxin, RANTES, macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein-3 (MCP-3), MCP-4 and IL-8] are chemotactic for eosinophils *in vitro and/or* induce the accumulation of eosinophils at sites of administration *in vivo*. These molecules may also have important roles in regulating the accumulation of eosinophils at sites of allergic inflammation (Rot *et al.*, 1992; Dahinden *et al.*, 1994; Santz *et al.*, 1995; Milne *et al.*,

1995; Lukacs *et al.,* 1996a; Alam *et al.,* 1996; Garcia *et al.,* 1996a, 1996b; White *et al.,* 1997).

I.4.4.3.1 *Non-chemokine cytokines.*

I.4.4.3.1.1 *IL-1 and IL-2.*

IL-1β is rapidly produced in the nasal mucosa of individuals with atopic rhinitis following allergen exposure and elevated levels of this cytokine are also found in the skin lesions of patients with atopic dermatitis (Bochner *et al.,* 1990; Bachert and Ganzer, 1993). IL-1 (subtype undefined) also plays an important role in the induction of pulmonary eosinophilia during allergic inflammation in guinea pigs (Watson, 1993). Administration of recombinant murine IL-1β to the skin of rats leads to a dose dependent accumulation of In^{111}-labelled eosinophils (Sanz *et al.,* 1995). The ability of IL-1β to induce the accumulation of eosinophils *in vivo* may involve the generation of secondary molecules such as PAF and IL-8, as PAF antagonist and anti-IL-8 mAb inhibited IL-1β-induced accumulation of eosinophils in the skin of rats. *In vitro,* IL-1β induces the migration of eosinophils across cultured endothelial cells and may regulate migration by activating adhesion systems (Ebisawa *et al.,* 1992).

IL-2 is a potent *in vitro* chemotactic agent for human eosinophils expressing CD25 (IL-2 receptor) (Rand *et al.,* 1991). This cytokine may also promote the accumulation of eosinophils in tissues, although no role for this molecule in this capacity could be demonstrated in a murine model of antigen-induced eosinophilic inflammation (Hom and Estridge, 1994). Milne *et al.,* (1995) reported that the exposure of guinea pigs to an aerosol of IL-2 caused an elevation in the levels of both eosinophils and macrophages in the BALF 24 hours later. These investigators also demonstrated, with the use of anti-IL-5 mAb, that this response was IL-5 independent, unlike earlier studies in mice (Yamaguchi *et al.,* 1990). Other experiments examining the effects of IL-2 administration on the accumulation of eosinophils in guinea-pig skin failed to show any effect over a period of 3 hours suggesting that IL-2 does not act as an eosinophil chemoattractant.

I.4.4.3.1.2 *IL-4.*

IL-4 is chemotactic for eosinophils that are isolated from the blood of atopics but not from the circulation of normal individuals and the intraperitoneal and intradermal administration of IL-4 in mice leads to the selective accumulation of eosinophils at sites of installation (Moser *et al.,* 1993; Dubois *et al.,* 1994). In a murine model of allergic airways eosinophilic inflammation, IL-4 deficiency was shown to selectively reduce the

accumulation of eosinophils in pulmonary tissues in comparison to wild type animals (Brusselle *et al.,* 1994).

IL-4 also potently and selectively induces transendothelial migration of "primed" eosinophils, but not neutrophils, *in vitro* and this effect maybe mediated by the upregulation of the CD11a/CD18, CD11b/CD18 and VLA-4 adhesion systems (Moser *et al.,* 1992a). IL-4 has also been shown to selectively up regulate the expression of VCAM-1, but not ICAM-1 or E-selectin on isolated endothelial cells. These results further support the concept that this cytokine may participate in the selective migration of eosinophils (but not neutrophils) into inflamed tissues (Schleimer *et al.,* 1992; Nakajima *et al.,* 1994).

Experiments in IL-4-deficient mice have also shown a role for this molecule in eosinophil trafficking *in vivo*. The accumulation of eosinophils in the airways of these mice was impaired during allergic airways inflammation in comparison to wild type mice (Hogan *et al.,* 1997), although, interestingly, ~~the migration of eosinophils into lung tissues and~~ the development of blood eosinophilia was unaffected. This suggests that IL-4 may promote eosinophil migration into the airways.

I.4.4.3.1.3 *TNF-α.*

TNF-α induces the migration of human eosinophils (but not neutrophils) *in vitro*, although, this response is through chemokinetic (random movement) rather than chemotactic (directional movement) mechanisms (Nagata *et al.,* 1993). There is conflicting evidence on the ability of TNF-α to induce the spontaneous transmigration of eosinophils through monolayers of HUVEC. TNF-α induced the migration of both eosinophils and neutrophils through naked filters *in vitro*, however, only neutrophils migrated through these filters when they were covered with a monolayer of HUVEC (Bittleman *et al.*, 1996). In contrast, other studies (Ebisawa *et al.*, 1992) showed that TNF-α induced the migration of eosinophils through monolayers of HUVEC. Elevated levels of TNF-α are present in the BALF of asthmatics and recently it was demonstrated that the instillation of recombinant guinea-pig TNF-α in to the lungs of guinea pigs induced a delayed pulmonary eosinophilia, 24-72 hours post administration. Together these observations suggest a role for TNF-α in the induction of pulmonary eosinophilia during allergic airways inflammation (Cembryznsha-Nowak *et al.,* 1993; White *et al.,* 1997).

The inhibition of TNF-α activity *in vivo* using a fusion protein (soluble TNF-α receptor linked to the Fc portion of antibody) reduced both neutrophil and eosinophil infiltration into the lungs of mice during antigen-induced airways inflammation (Lukacs *et al.,*

1995a). Similar experiments in guinea pigs also showed a role for TNF-α in eosinophil and neutrophil trafficking to sites of allergic inflammation (Renzetti *et al.*, 1996). TNF-α may promote eosinophil and neutrophil accumulation in tissues through the activation of the ICAM-1 and E-selectin adhesion pathways (Tonnel *et al.*, 1996). This cytokine, in synergy with IL-4, may also promote the selective accumulation of eosinophils at sites of allergic inflammation by activating the VCAM-1 adhesion system (Iademarco *et al.*, 1995).

I.4.4.3.1.4 *IL-3, GM-CSF and IL-5.*

IL-3, GM-CSF and IL-5 are eosinophil-trophic cytokines that also act as chemoattractants for normal human blood eosinophils *in vitro*, but not for eosinophils isolated from the blood of atopics (Sehmi *et al.*, 1992). Similar to TNF-α, the action of IL-5 on eosinophil migration *in vitro* is thought to be through the stimulation of chemokinesis rather than chemotaxis (Schweizer *et al.*, 1996). Elevated levels of IL-5, IL-3 and GM-CSF are present in the BALF and sera of both atopic and non-atopic asthmatics. Furthermore, the priming of normal human blood eosinophils with either IL-3 or IL-5 promotes enhanced chemotactic responses to PAF. Interestingly, the priming of eosinophils with IL-3 or IL-5 inhibits the migration of these cells in response to GM-CSF or IL-5 (Walker *et al.*, 1992; Warringa *et al.*, 1992a; 1992b; Sehmi *et al.*, 1992). Receptors for each of these cytokines share a common β-subunit and may be involved in cross (IL-3 and IL-5 on GM-CSF) or self desensitisation (IL-5 on IL-5) (Miyajima *et al.*, 1993).

Of these three cytokines only IL-5 selectively promotes the accumulation of eosinophils *in vitro* with IL-3 and GM-CSF also inducing the accumulation of basophils and neutrophils (Sehmi *et al.*, 1992; Yamaguchi *et al.*, 1992). The administration of IL-5 leads to the accumulation of eosinophils in both guinea-pig and human tissues at sites of administration (Terada *et al.*, 1992; Iwama *et al.*, 1993; Terada *et al.*, 1993; Van Oosterhout *et al.*, 1993). Elevated levels of IL-5 are found in the BALF of asthmatics following segmental allergen challenge and these levels often correlate with increased levels of eosinophils in the BALF (Sur *et al.*, 1996). In various animal models of allergic inflammation, mAbs against IL-5 also reduce the accumulation of eosinophils in tissues at sites of allergen provocation (Gulbenkian *et al.*, 1992; Mauser *et al.*, 1993; Kurup *et al.*, 1994). It should be noted that while anti-IL-5 mAb reduced the accumulation of eosinophils in inflamed tissues during allergic responses, many of these animals also exhibit reduced circulating eosinophil levels following antibody treatment. This indicates that the suppression of tissue eosinophilia may not be through the inhibition of eosinophil chemotaxis, but due to the reduction in the available blood pool of this cell.

IL-5 has pleotrophic actions on eosinophil function, regulating eosinophil differentiation, chemotaxis and survival. Thus, in many of these studies it may also be possible that the anti-IL-5 mAbs are inhibiting the differentiation of eosinophils in the bone marrow and the subsequent generation of blood eosinophilia. The immediate action of anti-IL-5 mAb in the inhibition of the accumulation of eosinophils in some of these studies, however, does suggest a role for IL-5 in eosinophil migration. Whether IL-5 acts directly as an eosinophil chemoattractant *in vivo* or stimulates the generation of a second messenger is yet to be established.

Notably, IL-5 deficiency greatly reduces pulmonary eosinophilia in a mouse model of allergic airways inflammation, but does not totally inhibit accumulation (Foster *et al.*, 1996). Once again, this may reflect the lower circulating levels of eosinophils in these mice and their inability to mount a blood eosinophilia following multiple exposures to aeroallergen, rather than as a result of impaired eosinophil chemotaxis at sites of inflammation (Foster *et al.*, 1996).

I.4.4.3.2 *Role of chemokines in eosinophil trafficking.*

Recent and exciting developments in the elucidation of the mechanisms regulating eosinophil trafficking at sites of inflammation have implicated numerous chemokines (Reviewed by Kita and Gleich, 1996). Chemokines are a super family of low molecular weight cytokines (6-14 kDa) that share a degree of amino acid homology and are chemotactic for leukocytes. Chemokines are divided primarily into one of two families, C-X-C (also know as α) or C-C (also known as β) depending upon the proximity of the first two of four highly conserved cysteine residues within the amino acid sequence. More recently a third family of chemokines was defined with the discovery of IL-16 (also know as lymphotaxin) that does not contain the C-C or C-X-C structural motifs.

The C-X-C family of chemokines is typified by IL-8 (Cohen and Cohen, 1996). This chemokine has a broad range of effector functions on neutrophils. However, IL-8 is also chemotactic for human eosinophils *in vitro*, although it appears to be active only on cells isolated from atopics. This may reflect priming of eosinophils in these individuals since this cell from normal individuals only become responsive to IL-8 following exposure to IL-5, IL-3 or GM-CSF (Warringa *et al.*, 1991; Schweizer *et al.*, 1994).

Members of the C-C chemokine family include MIP-1α and β, MCP-1, -2, -3 (and more recently MCP-4 and -5), RANTES and eotaxin. These molecules are typically chemotactic for monocytes, basophils, eosinophils and some T-lymphocyte subsets, but not for neutrophils (Kameyoshi *et al.*, 1992; Dahinden *et al.*, 1994; Liles and Van Voorhis, 1995). Of these chemokines, MCP-3, -4 and -5, RANTES, eotaxin and to a

lesser extent MIP-1α and MCP-2, are chemotactic for eosinophils and may function to promote eosinophil trafficking into inflamed tissues during allergic responses (Rot *et al.*, 1992; Dahinden *et al.*, 1994; Jose *et al.*, 1994a; Baggiolini and Dahinden, 1994; Baggiolini, 1996). Many of these chemokines can signal through common receptors expressed on the eosinophil and have the ability to self- and cross- desensitise (Dahinden *et al.*, 1994; Noso *et al.*, 1994).

I.4.4.3.2.1 *Macrophage inflammatory protein-1α.*

MIP-1α is chemotactic for normal human eosinophils *in vitro*, although this molecule is not as potent as RANTES or C5a (Rot *et al.*, 1992). Although elevated levels of MIP-1α are found in the BALF of atopic asthmatics, anti-MIP-1α mAb did not inhibit BALF-induced eosinophil chemotaxis *in vitro* (Alam *et al.*, 1996). MIP-1α is a potent chemoattractant for murine eosinophils isolated from IL-5 transgenic mice and a role for this chemokine in the accumulation of eosinophils in a murine model of allergic airways inflammation has been demonstrated (Lukacs *et al.*, 1995b; Rothenberg *et al.*, 1996). The pretreatment of antigen-sensitised mice with anti-MIP-1α mAb inhibited the accumulation of eosinophils in allergic lungs by half (Lukacs *et al.*, 1995b). Furthermore, the chemotactic activity of homogenates of antigen sensitised mouse lungs was partially inhibited by anti-MIP-1α mAb (Lukacs *et al.*, 1996b). Thus, MIP-1α may be an important chemoattractant for murine eosinophils *in vivo*.

Interestingly, while MIP-1α is not as potent as RANTES at inducing the chemotaxis of human eosinophils *in vitro,* this chemokine is far more potent than RANTES at inducing changes in transient intracellular free calcium levels in eosinophils (Rot *et al.*, 1992). Furthermore, RANTES was shown to desensitise the effect of MIP-1α on changes in intracellular calcium levels in eosinophils, although, interestingly MIP-1α did not desensitise eosinophils to RANTES. Other groups have shown that MIP-1α and RANTES cross desensitised eosinophils to each other (Van Riper *et al.*, 1994). MIP-1α and RANTES are ligands for C-C chemokine receptor 1 (CCR1) and CCR3, both of which are expressed on the surface of human eosinophils (Combadiere *et al.*, 1995a). Discrepancies between the findings of these groups could be due to species differences and the level of expression of CCRs. MIP-1α may also have other actions on eosinophil function besides the induction of chemotaxis. Recently, MIP-1α was shown to induce the production of reactive oxygen species by eosinophils (Kapp *et al.*, 1994).

I.4.4.3.2.2 *Monocyte chemoattractant protein-2, -3, -4 and -5.*

MCPs 2 to 5 are all chemotactic for human eosinophils *in vitro* with varying efficacy. Early studies showed that MCP-3 and to a lesser extent MCP-2, induces the migration of

eosinophils in modified Boyden chambers *in vitro*. These studies also demonstrated that MCP-2 and -3 and RANTES may signal through the same receptor(s) and cross desensitise respective responses on human eosinophils (Noso *et al.*, 1994). MCP-3 and RANTES may signal through two types of CCRs expressed on eosinophils (Dahinden *et al.*, 1994) and subsequent reports have shown that these molecules both signal through CCR1 and CCR3 (Combadiere *et al.*, 1995b; Ponath *et al.*, 1996a). Investigations have indicated a role for MCP-3 in the accumulation of eosinophils during allergic inflammation in both humans and mice. MCP-3 mRNA expression was elevated in the skin of atopics, but not normal individuals following allergen provocation at this site and correlated with the dermal infiltration of eosinophils (Ying *et al.*, 1995). Elevated levels of cells expressing MCP-3 mRNA are also found in the lungs of both atopic and non-atopic symptomatic asthmatics relative to non-asthmatic controls (Humbert *et al.*, 1997). Furthermore, the *in vitro* eosinophil chemotactic activity of BALF obtained from allergic asthmatics is partially inhibited by anti-MCP-3 mAb (Alam *et al.*, 1996). Collectively these data suggest a role for this chemokine in eosinophil trafficking during allergy.

The recent discovery of MCP-4 (human) and MCP-5 (mouse) and the demonstration that they are chemotactic for eosinophils indicates a possible role for these molecules in the accumulation of eosinophils in inflamed tissues during allergy (Uguccioni *et al.*, 1996; Garcia *et al.*, 1996a; Jia *et al.*, 1996). Although a role for MCP-4 in the accumulation of eosinophils *in vivo* has yet to be demonstrated, MCP-5 appears to be involved in eosinophil trafficking in mice. Airways eosinophilia during allergic inflammation in mice was significantly reduced by anti-MCP-5 mAb treatment (Jia *et al.*, 1996). Further investigations will be necessary to dissect the mechanisms of action of these molecules in eosinophil trafficking.

I.4.4.3.2.3 *RANTES*.

Regulated upon activation in normal T-cells expressed and secreted or as more commonly know RANTES, is a potent *in vitro* chemoattractant for eosinophils and memory type T-lymphocytes (Kameyoshi *et al.*, 1992). Elevated levels of this chemokine are present in the BALF of atopic asthmatics and the *in vitro* eosinophil chemotactic activity of the BALF from these patients is partially inhibited by anti-RANTES mAb (Alam *et al.*, 1996). The intradermal administration of human RANTES also leads to a localised eosinophilia in dogs and Rhesus monkeys, but not in guinea pigs. This suggests that the action of this molecule on eosinophil chemotaxis is species specific (Meurer *et al.*, 1993; Jose *et al.*, 1994a; Ponath *et al.*, 1996b).

Elevated mRNA levels for RANTES were seen in the BALF of antigen sensitised mice following antigen provocation in a model of allergic airways inflammation and correlated

with the accumulation of eosinophils in pulmonary tissues (Gonzalo *et al.*, 1996a). In similar studies, the chemotactic activity of the BALF from antigen sensitised and challenged mice was inhibited *in vitro* in the presence of anti-RANTES mAb (Lukacs *et al.*, 1996b). Increased numbers of cells expressing RANTES mRNA have been observed in atopic and non-atopic asthmatics, relative to non-asthmatic controls (Humbert *et al.*, 1997). Other studies, however, failed to show a correlation between the elevated RANTES mRNA levels and the accumulation of eosinophils at skin sites of allergic inflammation (Ying *et al.*, 1995).

I.4.4.3.2.4 *Eotaxin.*

Eotaxin is a novel eosinophil active C-C chemokine that is approximately 8.4 kDa in size. This chemokine was originally identified in the BALF from actively sensitised guinea pigs following antigen provocation (Jose *et al.*, 1994a). Eotaxin potently induces the selective accumulation of In^{111}-labelled eosinophils when injected intradermally and is a selective inducer of pulmonary eosinophilia when administered by aerosol to guinea pigs (Griffiths-Johnson *et al.*, 1993; Jose *et al.*, 1994a). Murine and human homologues have since been characterised and both are potent eosinophil chemoattractants *in vitro* (Rothenberg *et al.*, 1995a; Ponath *et al.*, 1996b).

Murine eotaxin has been shown to be chemotactic for eosinophils, but not monocytes/macrophage, lymphocytes or neutrophils, *in vitro* (Rothenberg *et al.*, 1995a). The accumulation of eosinophils in the skin of guinea pigs was amplified by the intravenous administration of IL-5 one hour prior to the intradermal injection of eotaxin (Collins *et al.*, 1995). It was suggested that IL-5 indirectly enhanced the chemotactic action of eotaxin by inducing a peripheral blood eosinophilia.

In contrast to experiments in guinea pigs, the instillation of eotaxin in the lungs of naive mice did not induce a pronounced accumulation of eosinophils in the airways, however, the instillation of eotaxin in the lungs of mice that are transgenic for the IL-5 gene induced a strong and selective pulmonary eosinophilia (Rothenberg *et al.*, 1996). This indicates that IL-5 and eotaxin act cooperatively to traffic eosinophils into tissues. IL-5 may in part promote eosinophil accumulation by enhancing the available blood eosinophil pool as IL-5 transgenic mice exhibit a pronounced blood eosinophilia (Tominaga *et al.*, 1991). Eotaxin mRNA is constitutively expressed at low levels in many tissues of mice and guinea pigs (Rothenberg *et al.*, 1995b; Gonzalo *et al.*, 1996b). Increased mRNA expression of this chemokine is seen in the lungs of sensitised mice and guinea pigs following aeroallergen exposure (Rothenberg *et al.*, 1995b). In sensitised mice, the kinetics of eotaxin mRNA expression in the lungs parallels the recruitment of eosinophils into this tissue following aeroallergen exposure (Gonzalo *et al.*, 1996b) and recently,

eotaxin was shown to regulate early eosinophil trafficking into allergic inflamed tissues in mice (Rothenberg, *et al.*, 1997). Naive guinea pigs exhibit a higher constitutive level of eotaxin mRNA expression than mice, furthermore, guinea pigs also have a higher basal level of pulmonary residing eosinophils than mice, suggesting that this molecule may regulate basal level eosinophil trafficking (Rothenberg *et al.*, 1995a, 1995b; Matthews *et al.*, 1998). Eotaxin may also maintain the basal circulating eosinophil pool, since eotaxin deficient mice have reduced levels of eosinophils in the blood (Matthews *et al.*, 1998).

Human eotaxin is as potent as RANTES at inducing human eosinophil chemotaxis *in vitro*, however, human eotaxin is much stronger than RANTES at inducing the accumulation of eosinophils when administered intradermally to Rhesus monkeys (Ponath *et al.*, 1996b).

The potent and selective action of eotaxin on eosinophil migration suggests an important role for this molecule in eosinophil trafficking *in vivo*. Furthermore, eotaxin acts co-operatively with IL-5 suggesting that these two cytokines are key regulators of eosinophilia during allergic inflammation.

I.4.5 Mechanisms of eosinophil activation and degranulation.

Eosinophil activation and degranulation at sites of allergic inflammation are thought to be central to the pathogenesis of asthma and dermatitis (Seminario and Gleich, 1994). Upon activation, eosinophils may secrete numerous cytokines (IL-1α, IL-3, IL-4, IL-5, IL-6, TGF-α and β, GM-CSF and TNF-α), chemokines (IL-8, MIP-1α, eotaxin and RANTES) and lipid mediators (leukotriene C_4, PAF, 15-hydroxyeicosatetraenoic acid, prostaglandins E_1 and E_2 and thromboxane B_2) (reviewed in Wardlaw *et al.*, 1994; Moqbel *et al.*, 1994; Garcia *et al.*, 1996b). Many of these molecules have direct effects on eosinophil priming, activation, trafficking, survival and degranulation and may function to amplify the inflammatory response (Jose *et al.*, 1994a; Wardlaw *et al.*, 1994; Cohen and Cohen, 1996). Eosinophils are also a rich source of numerous cytotoxic cationic proteins (MBP, EDN, ECP and EPO) (Wardlaw *et al.*, 1995). These proteins are contained in the granules of the cell cytoplasm and in response to specific stimulus are released into the extracellular environment through a process termed degranulation. The stimulation of eosinophils may also lead to the generation of respiratory bursts which results in the release of reactive oxygen species that may contribute to tissue damage and the inflammatory process in atopic diseases such as asthma and dermatitis (Martin *et al.*, 1996)

I.4.5.1 *Molecules that are involved in eosinophil activation and degranulation.*

Eosinophil degranulation is induced *in vitro* in the presence of immobilised immunoglobulins (IgG, IgA, sIgA and IgE), cytokines (IL-5 and GM-CSF), chemokines (RANTES and MIP-1α), PAF, complement peptides (C3a and C5a), fMLP, melanin and substance P (Khaliffe *et al.,* 1985, 1986; Abu-Ghazaleh *et al.,* 1989; Kroegel *et al.,* 1989, 1990; Fujisawa *et al.,* 1990; Kita *et al.,* 1992, 1994, 1996; Rot *et al.,* 1992; Takafuji *et al.,* 1994; Hoire and Kita, 1994; Daffern *et al.,* 1995).

I.4.5.1.1 *The role of immunoglobulins in eosinophil activation and degranulation.*

Eosinophils isolated from atopic individuals undergo degranulation, releasing EPO, when incubated with specific antigen or anti-human IgE mAb (Tomassini *et al.*, 1991). Immobilised IgG1 or IgG3, but not IgE mAbs also induces the release of EDN from eosinophils (Kaneko *et al.*, 1995). Different immunoglobulin isotypes have been shown to release individual eosinophilic granule proteins into the extracellular environment and may provide a key signal for eosinophil degranulation. The incubation of eosinophils isolated from atopic individuals with anti-IgE mAb leads to the release of EPO, but not ECP (Tomassini *et al.*, 1991), while anti-human IgA induces the release of both EPO and ECP from these cells. In contrast, the incubation of eosinophils with anti-IgG mAbs preferentially trigger the release of ECP but not EPO. Thus, specific immunoglobulins may be involved in the selective release of specific eosinophil granule proteins, with the potential to illicit particular physiological effects *in vivo*.

Although *in vitro* studies indicate a role for immunoglobulins in eosinophil degranulation *in vivo* and that elevated levels of allergen specific immunoglobulins are present in the sera of atopics, airways dysfunction and by inference eosinophil degranulation, during allergic airways inflammation in mice occurred independently of this pathway (Hogan *et al.*, 1996). Some studies have shown a role for IgE in eosinophil mediated airways dysfunction following aeroallergen provocation (Hamelmann *et al.*, 1997). In contrast, similar studies indicate that eosinophil mediated airways dysfunction occurs in IgE deficient mice (Mehlhop *et al.*, 1997).

I.4.5.1.2 *Immunoglobulin-independent regulation of eosinophil degranulation and activation.*

Although IL-5, IL-3 and GM-CSF promote eosinophil degranulation, their role is in the priming of eosinophils for events leading to degranulation in response to immunoglobulin stimuli (Fugisawa *et al.,* 1990). IL-3, IL-5 and GM-CSF are released at sites of

inflammation and may enhance eosinophil survival during allergy through autocrine mechanisms and with regards to IL-5 and GM-CSF this process may involve the inhibition of eosinophil apoptosis (Rothenberg *et al.,* 1988; Stern *et al.,* 1992; Czech *et al.,* 1993; Adashi *et al.,* 1995; Hu, 1997).

Eosinophils may also be degranulated by non-immunoglobulin stimuli and this mechanism may first depend on the activation or priming of the cell. PAF induces the release of ECP from eosinophils isolated from the blood of atopics, but not normal individuals (Eda *et al.,* 1993; Turner *et al.,* 1994). In contrast to PAF, fMLP, C5a and C3a all induce the release of ECP from eosinophils from normal donors (Turner *et al.,* 1994). PAF stimulates normal donor eosinophils to release EDN *in vitro*, as does recombinant human GM-CSF (Hoire and Kita, 1994). Interestingly, two cationic proteins that are released from eosinophils during degranulation (MBP and EPO) also potently induce non-cytolytic eosinophil degranulation (Kita *et al.*, 1995). This suggests that eosinophil degranulation at sites of allergic inflammation may be amplified by autocrine mechanisms.

I.4.5.1.3 *A role for adhesion molecules in eosinophil degranulation.*

Adhesion molecules may play a significant role in eosinophil degranulation. The degranulation of eosinophils by PAF or GM-CSF *in vitro* requires eosinophil adhesion and is inhibited by anti-CD11a/CD18 (Mac-1) mAb. The binding of eosinophils to fibronectin augments degranulatory responses to fMLP *in vitro*. Eosinophil degranulation by fMLP requires cell adhesion to fibronectin and this is mediated by VLA-4 (Neeley *et al.,* 1994). Interestingly, PMA-induced eosinophil degranulation does not require the eosinophil to be adherent (Hoire and Kita, 1994).

Unlike eosinophils in the circulation, those present in the sputum of atopic asthmatics express ICAM-1 (Hansel *et al.,* 1991). ICAM-1 expression is induced on eosinophils following exposure to either IL-3, IL-5 or GM-CSF, in combination with TNF-α and these cytokines are present in the BALF of atopic asthmatics (Hansel *et al.,* 1991; Czech *et al.,* 1993). Recently, the expression of ICAM-1 on eosinophils was shown to be important for the stimulation of eosinophil degranulation [as measured by EPX (EDN) release] by GM-CSF and TNF-α (Hoire *et al.*, 1997). In this study, the addition of anti-CD18 mAb abrogated both eosinophil adhesion to human serum albumin coated tissue culture plates and GM-CSF and TNF-α-stimulated eosinophil degranulation. Furthermore, both cell aggregation and degranulation, but not adhesion was shown to be dependent on ICAM-1. Thus, eosinophil-eosinophil or eosinophil-lymphocyte interaction via ICAM-1 may be required for eosinophil degranulation.

Investigations to date suggest that eosinophils are stimulated to degranulate via two pathways, one dependent and the other independent of immunoglobulins. Furthermore, both of these pathways contain mechanisms for the selective release of individual granule proteins.

I.5 SUMMARY.

Eosinophil activation is thought to underlie the aetiology of allergic diseases. It is clear that the regulation of eosinophil trafficking and degranulation is a very complex process. Many of the molecules that have been shown to exert regulatory effects on the recruitment of eosinophils are not solely specific for this leukocyte and may have important roles in other immune processes. Current literature indicates that cytokines and chemokines are key regulators of eosinophilia and that these molecules provide attractive targets for therapeutic intervention in allergic inflammatory disease. Increasing evidence suggests that IL-5 and eotaxin may selectively regulate eosinophil recruitment, particularly during allergy, although many of these studies fail to define the precise roles of these molecules in this process. Recent reports also indicate that these two cytokines may act co-operatively.

Two mechanisms underlie eosinophil degranulation that are immunoglobulin dependent and independent. Immunoglobulins in particular have been implicated in the regulation of eosinophil degranulation at sites of allergic inflammation, although an essential requirement for these molecules in this process remains controversial. Some molecules, such as IL-5 may play important role in both pathways of eosinophil activation . The elucidation of mechanisms that regulate eosinophil recruitment and activation, may provide new avenues for the improved management of allergic inflammatory diseases such as asthma.

SPECIFIC AIMS OF THIS THESIS

1. To examine the relationship between interleukin-5 and eotaxin in the regulation of blood and cutaneous eosinophilia in mice.

2. To determine the relationship between interleukin-5 and eotaxin in regulating pulmonary eosinophilia and their role in airways dysfunction in mice.

3. To dissect the requirement of interleukin-5 in eosinophil trafficking *in vivo*.

4. To dissect the role of interleukin-5 in eosinophil trafficking and function during allergy.

5. To dissect the role of eotaxin in eosinophil trafficking and function *in vivo* and to determine its role in eosinophilic responses during allergy.

CHAPTER II

THE RELATIONSHIP BETWEEN INTERLEUKIN-5 AND EOTAXIN IN THE REGULATION OF BLOOD AND CUTANEOUS EOSINOPHILIA IN MICE

II.1 INTRODUCTION.

The recruitment of eosinophils at sites of allergic inflammation is a complex process which potentially may be regulated by inflammatory cytokines (IL-1β, IL-3, IL-4, IL-5, GM-CSF and TNF-α) and chemokines (RANTES, MCP-3, MIP-1α and eotaxin). Despite this apparent complexity, it is clear from investigations with IL-5 deficient mice that the absence of IL-5 alone abolishes the pronounced tissue and blood eosinophilia normally generated by allergic responses and parasite infections (Kopf *et al.,* 1996; Foster *et al.,* 1996). Furthermore, of the cytokines implicated in modulating eosinophilic inflammation only IL-5 and eotaxin have been identified to selectively regulate eosinophil trafficking (Kay *et al.,* 1991; Jose *et al.,* 1994a; Rothenberg *et al.,* 1995a; Gonzalo *et al.,* 1996a). Currently, it is unknown which of these cytokines provides the essential signal for eosinophil homing and migration into tissues.

IL-5 has been identified as a central mediator in the regulation of eosinophilic inflammation and in the aetiology of asthma and allergic disease (Coffman *et al.,* 1989; Kay *et al.,* 1991; Hamid *et al.,* 1991; Robinson *et al.,* 1992; Kopf *et al.,* 1996; Foster *et al.,* 1996). IL-5 not only regulates the growth, differentiation and activation of eosinophils (Owen *et al.,* 1987; Rothenberg *et al.,* 1988; Yamaguchi *et al.,* 1988; Lopez *et al.,* 1988; Clutterbuck *et al.,* 1989; Fujisawa *et al.,* 1990), but also provides an essential signal for the induction of eosinophilia during allergic inflammation (Coffman *et al.,* 1989; Kopf *et al.,* 1996; Foster *et al.,* 1996). Investigations with IL-5 deficient mice have established the blood and airways eosinophilia and the subsequent development of lung damage and airways hyperreactivity, that occurs in response to aeroallergen, is dependent on IL-5 (Foster *et al.,* 1996). However, these investigations also indicate that other factors derived from the site of antigen presentation are required to amplify the IL-5 signal for eosinophil migration and are essential for widespread eosinophilic inflammation in tissues.

Eotaxin, a member of the C-C branch of chemokines, has been recently identified as a novel chemotactic agent for eosinophils (Jose *et al.,* 1994a; Rothenberg *et al.,* 1995a). The potency and rapid action of eotaxin at inducing selective pulmonary and intradermal eosinophil recruitment suggests an integral role for this protein in the early phases of the signalling mechanism for eosinophil homing and tissue recruitment (Jose *et al.,* 1994b; Rothenberg *et al.,* 1995a; Gonzalo *et al.,* 1996b). Eotaxin is constitutively expressed in a number of tissues and may regulate basal tissue homing of eosinophils. Increased production of eotaxin in response to antigen-stimulation, in association with the increased synthesis of cytokines and eosinophil chemoattractant chemokines, may regulate eosinophil tissue homing and accumulation to the site of inflammation (Jose *et al.,* 1994b; Rothenberg *et al.,* 1995a; Gonzalo *et al.,* 1996b).

Investigations with guinea pigs suggest that eotaxin and IL-5 may act cooperatively to promote the recruitment of eosinophils into tissues (Collins *et al.,* 1995). The number of eosinophils recruited to sites of intradermal injection of eotaxin correlated with an increase in the number of circulating eosinophils induced by the systemic administration of IL-5. In mice, eotaxin-induced recruitment of eosinophils to the lung and skin was only consistently observed in IL-5 transgenic mice, which have elevated levels of IL-5 and a pronounced basal blood eosinophilia (Rothenberg *et al.,* 1996). Thus, during the inflammatory response, IL-5 may provide the signal for the release of a pool of eosinophils from the bone marrow, while eotaxin may elicit the signal for eosinophil localisation to the site of inflammation (Collins *et al.,* 1995; Rothenberg *et al.,* 1996). However, it is not clear whether the amplification of eotaxin-induced eosinophilia in the presence of increased levels of systemic IL-5 is directly due to this cytokine or to the concomitant increase in the number of circulating eosinophils.

In order to provide important insight into the molecular mechanisms regulating eosinophil homing and selective eosinophil trafficking during complex tissue responses to inflammatory stimuli, it is essential to gain a better understanding of the interaction between IL-5 and eotaxin. In order to further elucidate the relationship between IL-5 and eotaxin in eosinophil trafficking *in vivo,* these cytokines were assayed for their ability to induce tissue eosinophilia in skin, singularly or in combination, using a skin bioassay technique. In addition, these two cytokines were injected intravenously to determine their effect on circulating eosinophil levels. In this chapter, results suggest that eotaxin may have an important role in regulating blood eosinophilia and may act in synergy with IL-5 to promote the selective accumulation of eosinophils into tissues.

II.2 MATERIALS AND METHODS.

II.2.1 Animals.

Male mice (C57BL/6, 6-8 weeks of age) were used in all bioassay experiments.

II.2.2 Effect of i.v. IL-5 and/or eotaxin on circulating levels of eosinophils.

Mouse recombinant IL-5 was a generous gift from Professor Ian Young (John Curtin School of Medical Research, Australian National University) and was expressed and purified from the baculovirus expression system (Ingley *et al.,* 1991). Mice were injected intravenously (i.v.) with eotaxin [0.03-2.4 nmol/kg (Pepro Tech Inc., Rocky Hill, NJ., USA.)], IL-5 (10-100 pmol/kg) or control vehicle [100 µl of 10 mM PBS/0.1% BSA (< 0.1 ng/mg endotoxin), (pH 7.4)]. In some experiments mice were given an i.v. co-injection of eotaxin (1.2 nmol/kg) and IL-5 (100 pmol/kg) (at these concentrations both cytokines induced a blood eosinophilia of similar magnitude). Blood samples were taken before, at 30 minutes and hourly after the i.v. injection of eotaxin, IL-5, eotaxin with IL-5 or control vehicle for quantification of eosinophils. Eosinophils/ml blood were determined by diluting blood 1/10 in Discombe's solution (Discombe, 1946) and counting positively staining cells 10 minutes later by light field microscopy using a haemocytometer.

II.2.3 Effect of i.v. IL-5 and/or eotaxin on bone marrow levels of eosinophils.

Mice were injected i.v. with IL-5 (100 pmol/kg), eotaxin (2.4 nmol/kg) or control vehicle [100 µl of 10 mM PBS/0.1% BSA (< 0.1 ng/mg endotoxin) (pH 7.4)]. At the peak of the blood eosinophilia (30 minutes for both IL-5 and eotaxin) mice were sacrificed by cervical dislocation and the right femurs removed into ice cold Hank's buffered salt solutions (HBSS). Epiphyses from both the proximal and distal ends of the femurs were removed and the bone marrow cavity lavaged with 2 mls of HBSS. The number of cells/ml of lavage fluid was determined by using a haemocytometer and 2×10^5 cells were cytocentrifuged and stained with Giemsa-May-Grunwald for differential cell counting. Routinely, 300-400 cells were counted per slide.

II.2.4 Bioassay of eotaxin- and/or IL-5- induced accumulation of eosinophils in skin.

Mice were anaesthetised with ether, the dorsal skin shaved and then injected i.v. with IL-5 (100 pmol/kg) or control vehicle [100 µl of 10 mM PBS/0.1% BSA (< 0.1 ng/mg endotoxin) (pH 7.4)]. In some experiments mice were injected i.v. with eotaxin (1.2 nmol/kg) or in combination with IL-5 (100 pmol/kg). After 1 hour mice were injected subcutaneously (s.c.) with 150 µl of air and the required concentration of eotaxin (1-80 pmol/site), IL-5 (0.1-10 pmol/site) or a combination of both cytokines [IL-5 (10 pmol/site) and eotaxin (5.0 pmol/site) at concentrations that induced maximal response (EC_{100})] or control vehicle [100 µl HBSS/0.01% BSA (< 0.1 ng/mg endotoxin) (pH 7.4)]. Two hours after the s.c. injection of cytokine(s) or control vehicle, mice were sacrificed by CO_2 asphyxiation and the dorsal skin membrane [subcutaneous fascia (Lawman, 1984), approximately 18 x 18 mm] below the air pouch excised directly onto a glass slide. Slides were allowed to air dry before being fixed with 100% methanol and stained with May-Grunwald-Giemsa for differential cell counting. Eosinophils/mm^2 were determined by counting 10 fields of view (x 250 magnification) with reference to a calibrated grid fitted to the microscope's optical lens. Blood samples were taken before i.v. injection of IL-5, 30 minutes after injection and hourly thereafter. Eosinophils/ml of blood were determined by the Discombe's method (see section II.2.2).

II.2.5 Determination of half maximal effective concentrations for eosinophil chemoattractants.

Dose response curves for the following eosinophil chemoattractants were performed using the skin bioassay technique (see section II.2.4) with the following modification. Mice were given an i.v. injection (Tail vein) of 100 pmol/kg IL-5 in 0.1% BSA/PBS (pH 7.4) 30 minutes (instead of 1 hour) prior to receiving a s.c. co-injection of 100 µl of 0.01% BSA/HBSS (pH 7.4) containing either MIP-1α [0.01-1 pmols (AUSTRAL biologicals, San Ramon, CA., USA.)], RANTES [0.01-10 pmols (R&D Systems, Minneapolis, MN., USA.)], LTB_4 [0.1-100 pmols (SIGMA Chemical Co., St Louis, MO., USA.)], PAF [0.1-100 pmols (Cayman Chemical Company, Ann Arbor, MI., USA.)] or VGSE [0.01-1000 pmols (A gift from Dr Caryl Hill, John Curtin School of Medical Research, Australian National University and was synthesised by the Biomolecular Resource Facility of the John Curtin School of Medical Research)] and 150 µl of air. Half maximally effective concentrations (EC_{50}) of these molecules were determined from their respective dose response curves. EC_{50} = the dose of chemoattractant required for half maximal recruitment of eosinophils into the sites of ~~installation~~ injection (above background level of eosinophil recruitment induced by control vehicle). EC_{50} of eotaxin and IL-5 were

determined from dose response curves generated in skin as described in section II.2.4 and were confirmed using the modified skin bioassay technique as described above. EC_{50} for IL-5 = 0.5 pmol and EC_{50} for eotaxin = 2.5 pmol.

II.2.6 Bioassay to determine the relationship between chemoattractants for the accumulation of eosinophils in the skin.

The relationship between various chemoattractants for the recruitment of eosinophils to the skin was analysed using the modified back patch bioassay (see section II.2.5). Briefly, mice received an i.v. injection of IL-5 [100 pmol/kg in 0.1% BSA/PBS (pH 7.4)] and 30 minutes later were s.c. co-injected with one of the following combinations of chemoattractants at their respective EC_{50} in 100 µl of 0.01 % BSA/HBSS (pH 7.4)+ 150 µl air. IL-5 (0.5 pmol) with either eotaxin (2.5 pmol), MIP-1α (62.5 fmol), RANTES (0.5 pmol), LTB4 (1.0 pmol), PAF (0.5 pmol) or VGSE (0.5 pmol); or eotaxin (2.5 pmol) with either PAF (0.5 pmol) or LTB_4 (1.0 pmol). The accumulation of eosinophils in the dorsal skin membrane was determined 2 hours later (see section II.2.4).

II.3 RESULTS.

II.3.1 Effect of intravenous IL-5 and eotaxin on circulating and bone marrow eosinophil levels.

Intravenously administered IL-5 induced a rapid and sustained increase in circulating eosinophils (figure II.1a). The peak was at the first time point of 30 minutes and eosinophil numbers fell to basal levels over 5 hours. This effect was ~~concentration~~ dose-dependent and a dose of 100 pmol/kg induced an eosinophilia that was equivalent to that observed during allergic pulmonary inflammation in mice (Foster *et al.*, 1996). The rapid action of i.v. IL-5 in guinea pigs has been associated with the mobilisation of a bone marrow pool of eosinophils (Collins *et al.*, 1995). This mechanism also appeared to be operating in the present experiments as the blood eosinophilia correlated with a fall in bone marrow eosinophil numbers (figure II.2).

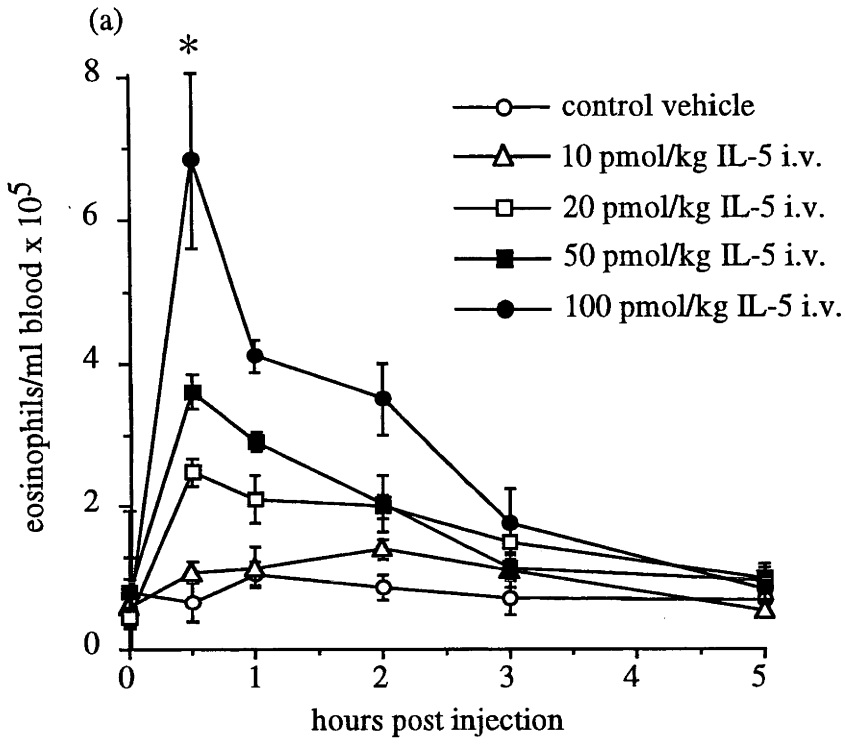
The effect of i.v. administration of eotaxin on circulating levels of eosinophils has not been previously reported. In this investigation i.v. eotaxin potently induced a dose (0.03-2.4 nmol/kg) dependent blood eosinophilia (figure II.1b). The onset of eosinophilia was extremely rapid and blood levels were at least equivalent to that observed during eosinophilic inflammation in mice (Foster *et al.*, 1996) and in response to i.v. IL-5 (figure II.1a). However, eotaxin was approximately 3 fold less potent than IL-5 in inducing an equivalent eosinophilia. Notably, the increase in blood eosinophil numbers in response to i.v. eotaxin (figure II.1b) did not correlate with a significant reduction in the level of bone marrow eosinophils (figure II.2a). The co-administration of both eotaxin (1.2 nmol/kg) and IL-5 (100 pmol/kg) induced a blood eosinophilia significantly greater than that induced by either of these two molecules alone (figure II.2b).

II.3.2 Effect of subcutaneously injected eotaxin and IL-5 on the accumulation of eosinophils in the skin.

In mice, as previously observed (Gonzalo *et al.*, 1996; Rothenberg *et al.*, 1996), eotaxin was a highly potent chemotactic signal for the selective and rapid recruitment of eosinophils to tissues. Within 2 hours, eotaxin (1.0-20 pmol/site) had induced a pronounced tissue eosinophilia which was highly dependent on concentration (figure II.3a). The effect of eotaxin was maximal at 5.0 pmol/site and subsequent increases in dose resulted in a concentration dependent reduction in the accumulation of eosinophils. Similar results were also obtained in mice with dilutions of crude supernatants from COS cells transfected with eotaxin cDNA and expressing eotaxin (Rothenberg *et al.*, 1995a).

Figure II.1 ***The effect of i.v. administration of IL-5 or eotaxin on circulating eosinophil numbers in mice.***

Mice were injected i.v. with IL-5 (10-100 pmol/kg), eotaxin (0.03-2.4 nmol/kg) or control vehicle [100 µl of 10 mM PBS/0.1% BSA (pH 7.4)]. Blood samples were taken before, at 30 minutes and hourly after i.v. injection of the cytokines or control vehicle for differential quantification of leukocytes. (a) IL-5 (10-100 pmol/kg) induced a dose dependent increase in circulating eosinophil levels. (b) Eotaxin (0.03-2.4 nmol/kg) induced a dose dependent increase in circulating eosinophil numbers. Results represent mean eosinophils/ml of blood ± SEM of groups of 5-6 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) * $P < 0.001$ compared with control vehicle at 30 minutes. (b) * $P < 0.001$ at 2.4 nmol/kg eotaxin compared with control vehicle.


(a)
*
8
6
4
2
0
eosinophils/ml blood x 10^5
control vehicle
10 pmol/kg IL-5 i.v.
20 pmol/kg IL-5 i.v.
50 pmol/kg IL-5 i.v.
100 pmol/kg IL-5 i.v.
0
1
2
3
4
5
hours post injection

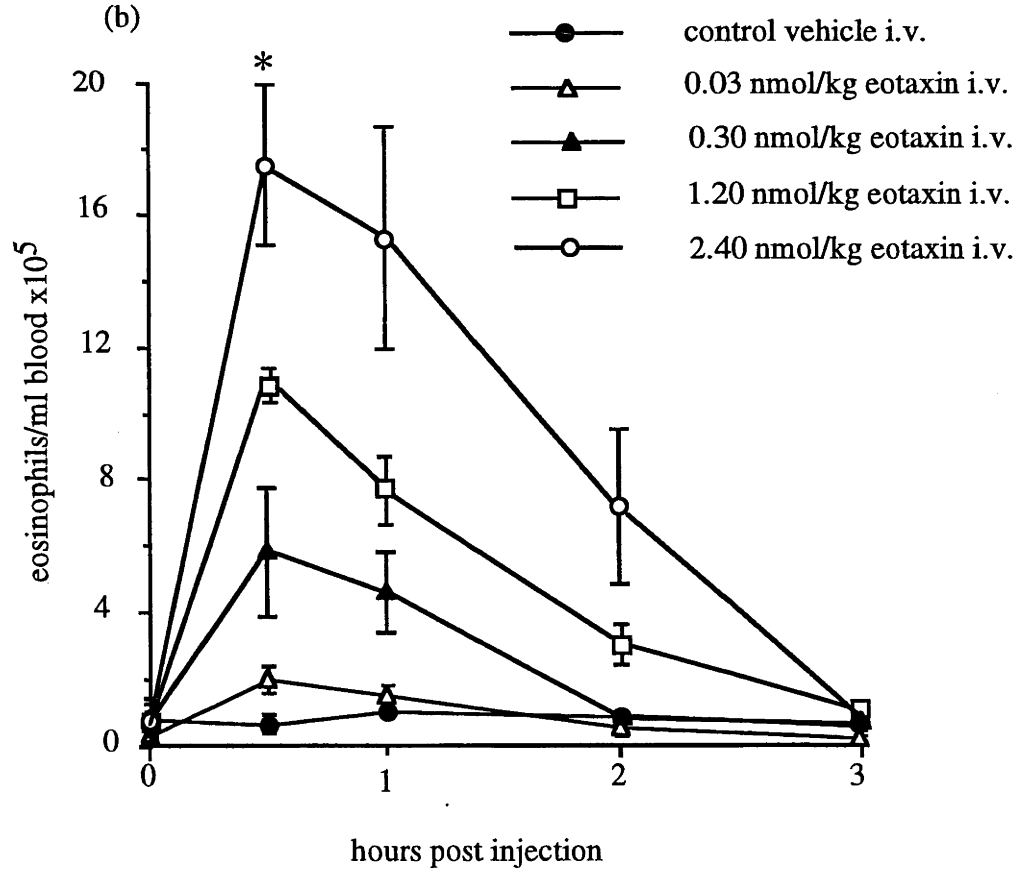

(b)
*
20
16
12
8
4
0
eosinophils/ml blood x10^5
control vehicle i.v.
0.03 nmol/kg eotaxin i.v.
0.30 nmol/kg eotaxin i.v.
1.20 nmol/kg eotaxin i.v.
2.40 nmol/kg eotaxin i.v.
0
1
2
3
hours post injection

Figure II.2 ***Eosinophil numbers in the bone marrow of mice in the presence of i.v. IL-5 or eotaxin and the effect of the co-administration of these two cytokines of circulating eosinophil levels.***

(a) The effect of i.v. injection of IL-5 (100 pmol/kg), eotaxin (1.2 nmol/kg) or control vehicle [100 µl of 10 mM PBS/0.1% BSA (pH 7.4)] on bone marrow levels of eosinophils. IL-5 but not eotaxin caused a reduction in bone marrow eosinophil numbers. Femurs were removed at the peak of the blood eosinophilic response (30 minutes) and the bone marrow cavity lavaged with 2 mls of HBSS. The number of cells/ml of lavage fluid was determined and 2 $x10^5$ cells were cytocentrifuged and stained with Giemsa-May-Grunwald for differential cell counting. Results represent the mean number of bone marrow eosinophils ± SEM for groups of 4 mice. (b) Mice were injected i.v. with IL-5 (100 pmol/kg), eotaxin (1.2 nmol/kg), both IL-5 (100 pmol/kg) and eotaxin (1.2 nmol/kg) or control vehicle [100 µl of 10 mM PBS/0.1% BSA (pH 7.4)] and the levels of eosinophil in the blood determined 30 minutes later. Mice that received IL-5 and eotaxin simultaneously i.v. had significantly higher eosinophil levels in the blood in comparison to treatment with an individual cytokine. Results represent mean eosinophils/ml of blood ± SEM of groups of 5-6 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) * $P < 0.001$ compared with control vehicle. (b) * $P < 0.001$ compared with IL-5 or eotaxin alone.

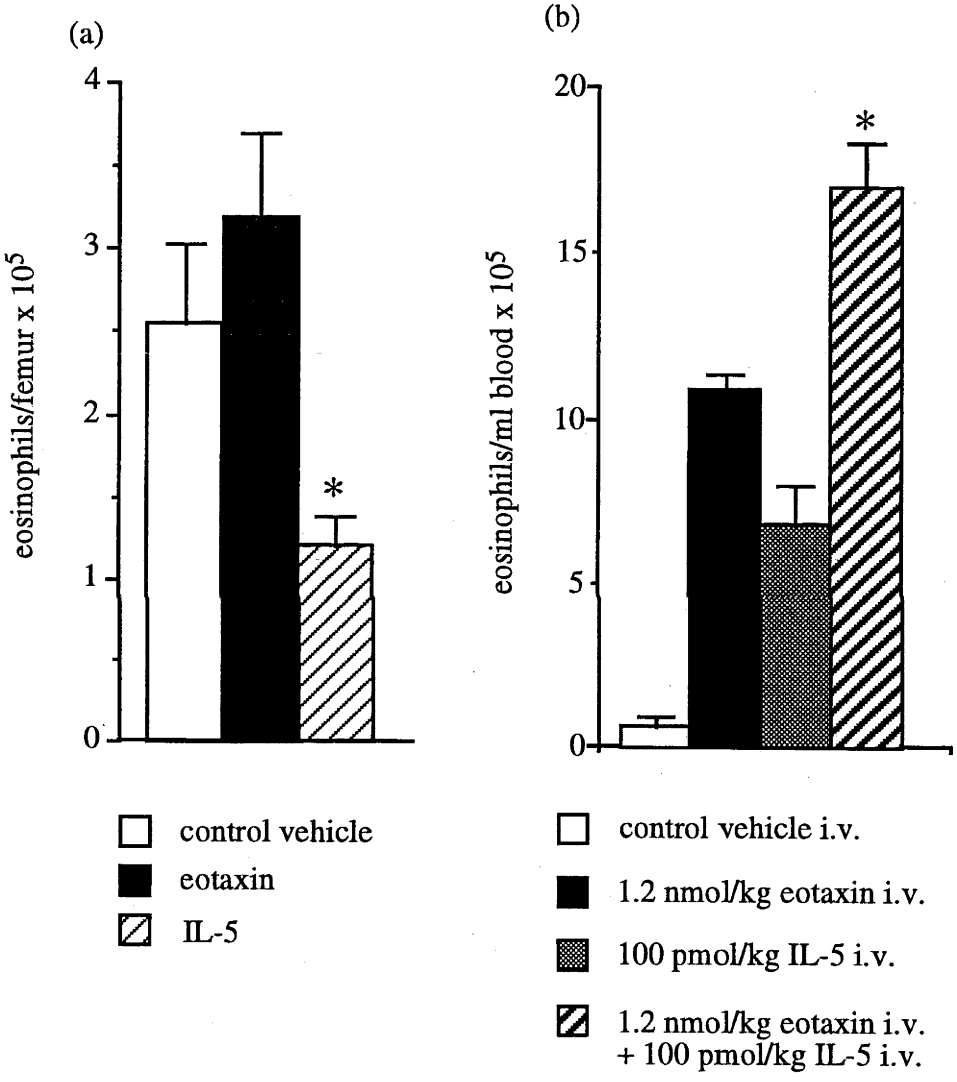
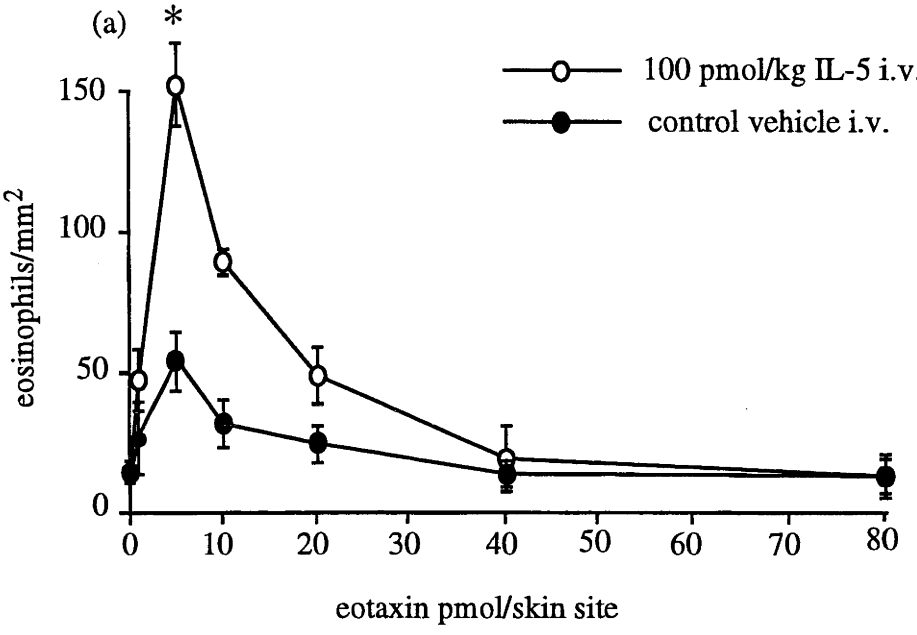
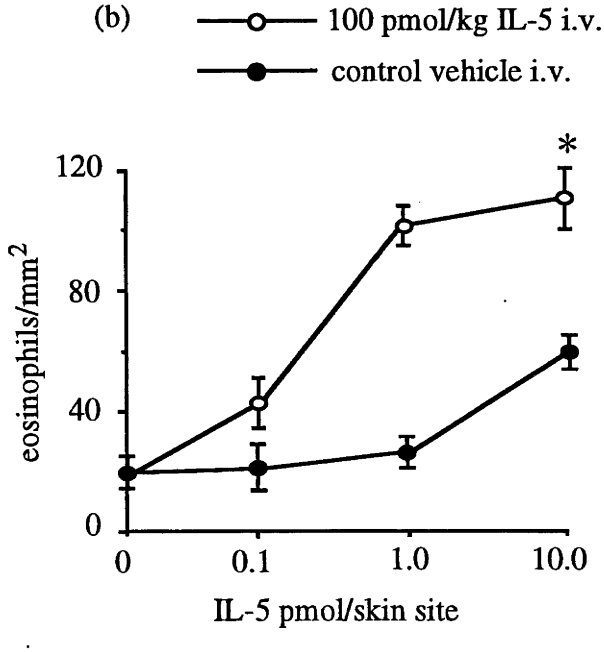

(a)
eosinophils/femur x 10^5
4
3
2
1
0
*
control vehicle
eotaxin
IL-5
(b)
eosinophils/ml blood x 10^5
20
15
10
5
0
*
control vehicle i.v.
1.2 nmol/kg eotaxin i.v.
100 pmol/kg IL-5 i.v.
1.2 nmol/kg eotaxin i.v.
+ 100 pmol/kg IL-5 i.v.

Figure II.3 ***The effect of i.v. IL-5 on eotaxin- and IL-5- induced eosinophil recruitment into the skin of mice.***

(a) Accumulation of eosinophils in dorsal skin membranes in response to subcutaneously injected eotaxin (1.0-80 pmol/site) or control vehicle [100 µl of HBSS/0.01% BSA (pH 7.4)]. Eotaxin-induced eosinophil recruitment (determined 2 hours after s.c. eotaxin administration) was concentration dependent and was determined in the presence and absence of i.v. IL-5. Mice were injected i.v. with IL-5 (100 pmol/kg) or control vehicle [100 µl of 10 mM PBS/0.1% BSA (pH 7.4)] and 1 hour later eotaxin or control vehicle was injected subcutaneously. Eosinophil recruitment was maximal at 5.0 pmol/site eotaxin. (b) Accumulation of eosinophils in dorsal skin membranes was determined as described above for (a), however, mice were subcutaneously injected with IL-5 (0.1 to 10.0 pmol/skin site) instead of eotaxin. IL-5-induced eosinophil recruitment was concentration dependent and was determined in the presence or absence of i.v. IL-5. Eosinophil recruitment was maximal at 10 pmol/site IL-5. Results represent mean eosinophils/mm^2 ± SEM of groups of 6 mice. One dorsal membrane preparation was excised from each animal and prepared for differential cell counting. Ten fields per preparation were counted for eosinophil infiltration and the mean obtained. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) * $P < 0.001$ compared to i.v. control vehicle at the same subcutaneous concentration of eotaxin. (b) * $P < 0.001$ compared to i.v. control vehicle at the same subcutaneous concentration of IL-5.


(a)
*
100 pmol/kg IL-5 i.v.
control vehicle i.v.
eosinophils/mm2
150
100
50
0
0
10
20
30
40
50
60
70
80
eotaxin pmol/skin site


(b)
100 pmol/kg IL-5 i.v.
control vehicle i.v.
*
eosinophils/mm2
120
80
40
0
0
0.1
1.0
10.0
IL-5 pmol/skin site

The effect of eotaxin on eosinophil transmigration was significantly amplified by first inducing a blood eosinophilia by i.v. injection of IL-5 (100 pmol/kg) (figure II.3a) 1 hour prior to eotaxin injection (corresponding blood levels are show in figure II.1a). These results, in part, may explain why some investigators see no or only poor chemotactic responses with eotaxin in the absence of systemic eosinophilia (Rothenberg *et al.,* 1996). Subcutaneously injected eotaxin or IL-5 did not induce a blood eosinophilia (results not shown). Interestingly, increased numbers of eosinophils in the blood in response to i.v. eotaxin did not significantly enhance responses to chemotactic stimuli in the skin (figure II.4b).

The subcutaneous injection of IL-5 (1-10 pmol/site) also induced a concentration dependent tissue eosinophilia which was also amplified by i.v. injection of IL-5 (figure II.3b). Tissue eosinophilia induced by IL-5 (10 pmol/site) was equivalent to that produced by eotaxin (5.0 pmol/site) (figure II.3a). The s.c. co-injection of eotaxin (5.0 pmol/site) and IL-5 (10 pmol/site) significantly amplified eosinophil recruitment at sites of administration in comparison to eotaxin or IL-5 alone (figure II.4a).

The relationship between the blood eosinophilia induced by eotaxin and/or IL-5 and the subsequent accumulation of eosinophils at s.c. sites of the administration of these chemoattractants was also examined (figure II.4). Interestingly, the i.v. injection of eotaxin (1.2 nmol/kg) induced a blood eosinophilia (figure II.1b), but did not amplify chemotactic responses to subcutaneously administered eotaxin (5.0 pmol/skin site) or IL-5 (10 pmol/skin site) (figure II.4b). In contrast, i.v. injection of IL-5 (100 pmol/kg) significantly potentiated the chemotactic responses of eosinophils to either s.c. administered cytokine (figure II.4b). Furthermore, the i.v. injection of a combination of both IL-5 (100 pmol/kg) and eotaxin (1.2 nmol/kg) significantly amplified chemotactic responses to s.c. eotaxin (5.0 pmol/skin site) or IL-5 (10 pmol/skin site) (figure II.4b). The accumulation of eosinophils in the skin in response to s.c. eotaxin or s.c. IL-5 in the presence of both i.v. eotaxin and IL-5 was significantly greater than responses obtained solely with i.v. IL-5.

II.3.3 The relationship between cytokines, chemokines, lipid mediators and chemotactic peptide in the accumulation of eosinophils in skin.

MIP-1α-, RANTES-, PAF-, LTB_4- and ECF-A tetrapeptide (VGSE)- induced dose-dependent accumulation of eosinophils in mouse skin (figures II.5 and II.6). At higher doses of most of these molecules, an inhibition of the accumulation of eosinophils occurred, indicating that like eotaxin the chemotactic action of these molecules *in vivo* is tightly regulated. Inhibition of eosinophil chemotaxis at high doses of VGSE *in vitro* has previously been reported (Boswell *et al.,* 1976).

Figure II.4 ***The effect of the s.c. co-administration of IL-5 and/or eotaxin on eosinophil recruitment into the skin and the effect of eotaxin and/or IL-5 i.v. on these eosinophilic responses in the skin.***

(a) Mice were injected i.v. with IL-5 (100 pmol/kg) and then 1 hour later injected s.c. with either IL-5 (10 pmol/site) or eotaxin (5 pmol/site) or with a combination of both cytokines. The co-injection of IL-5 and eotaxin induced accumulation of eosinophils significantly greater than either of these cytokines alone. (b) The effect of i.v. injection of IL-5 (100 pmol/kg) or eotaxin (1.2 nmol/kg) or a combination of both cytokines on the accumulation of eosinophils in dorsal skin membranes in response to s.c. eotaxin (5 pmol/site) or IL-5 (10 pmol/site). Maximal accumulation of eosinophils occurred in the presence of a combination of i.v. eotaxin and IL-5. Eotaxin, IL-5, eotaxin with IL-5 or control vehicle were injected into the skin 1 hours after i.v. administration of IL-5, eotaxin or control vehicle. Eosinophil recruitment into dorsal skin membranes was determined 2 hours after s.c. injection of eosinophil chemoattractants or control vehicle. Results represent mean eosinophils/mm^2 ± SEM of groups of 6 mice. One dorsal membrane preparation was excised from each animal and prepared for differential cell counting. Ten fields per preparation were counted for eosinophil infiltration and the mean obtained. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) * $P < 0.05$ compared to eotaxin or IL-5 alone and (b) * $P < 0.01$ compared to control vehicle i.v. and ** $P < 0.05$ compared to i.v. IL-5. No significant difference was observed between i.v. control vehicle and i.v. eotaxin groups, in the presence of s.c. eotaxin or IL-5.

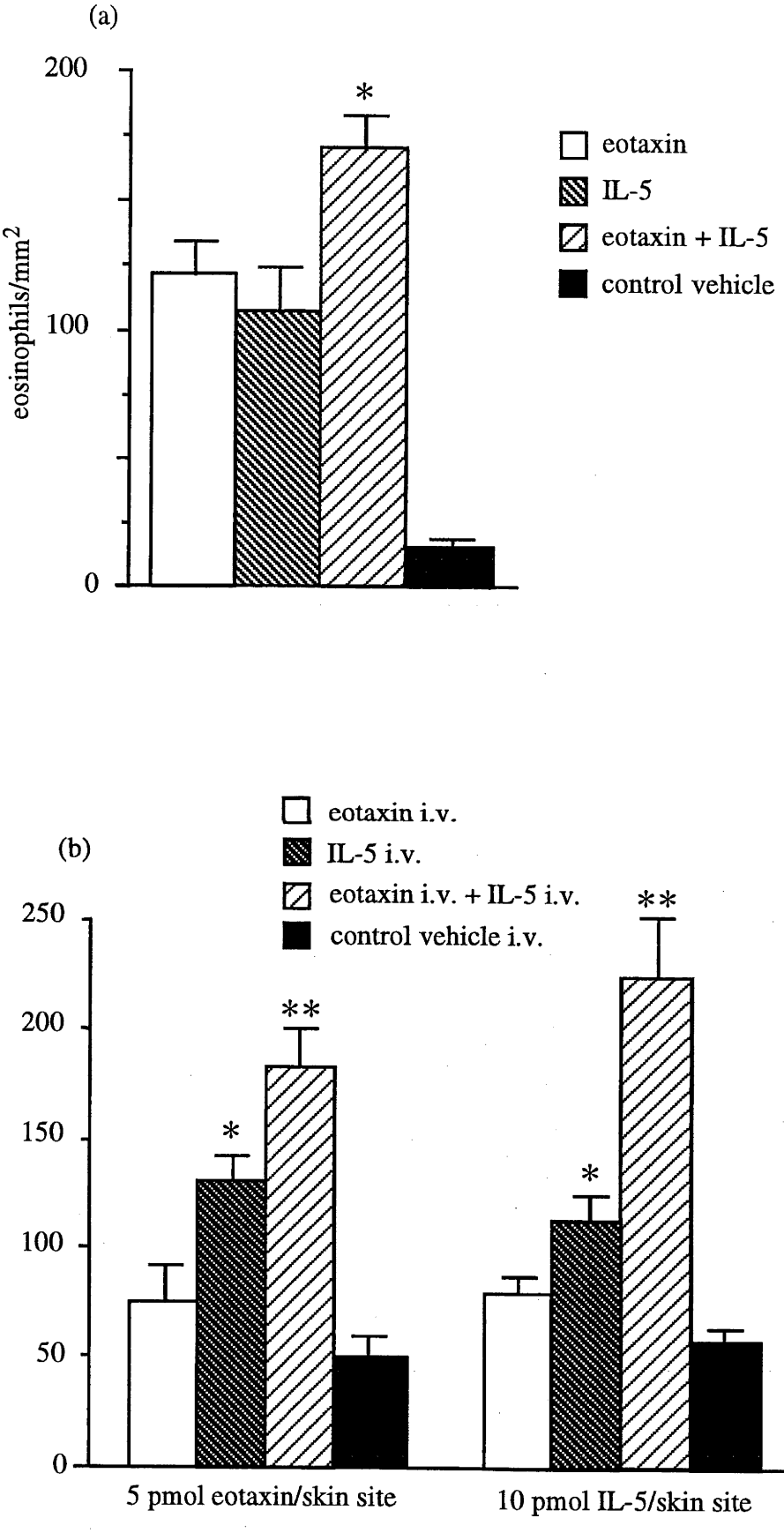
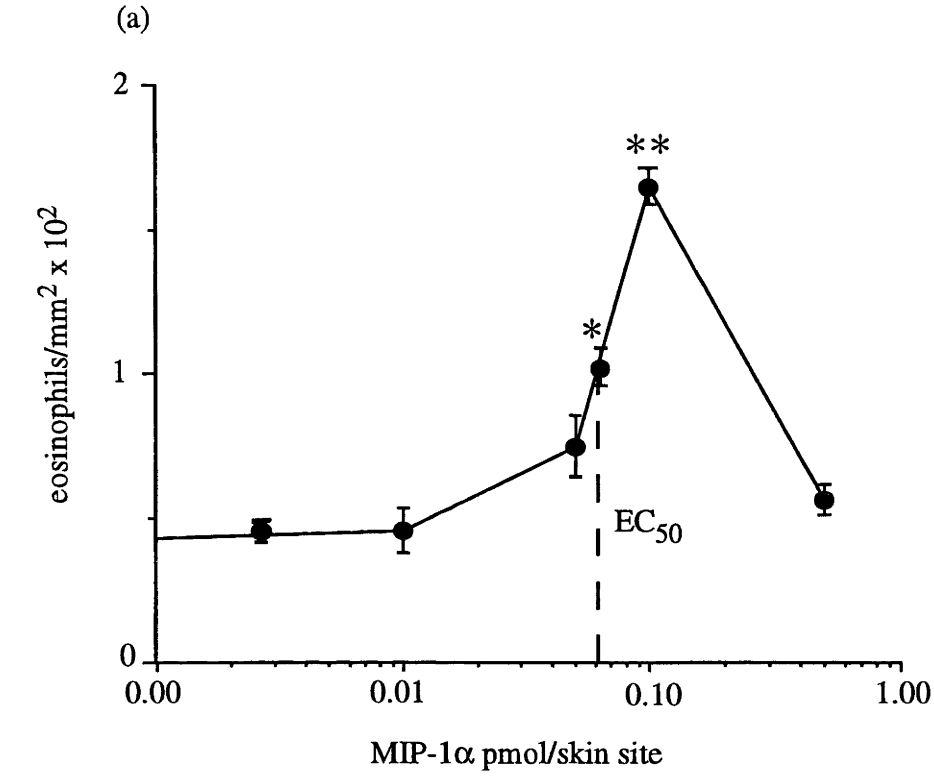

(a)
200
100
0
eosinophils/mm2
*
eotaxin
IL-5
eotaxin + IL-5
control vehicle
(b)
eotaxin i.v.
IL-5 i.v.
eotaxin i.v. + IL-5 i.v.
control vehicle i.v.
250
200
150
100
50
0
eosinophils/mm2
*
**
*
**
5 pmol eotaxin/skin site
10 pmol IL-5/skin site

Figure II.5 ***Dose response curves for MIP-α and RANTES and determination of half maximally effective concentrations for the accumulation of eosinophils in skin.***

MIP-1α and RANTES potently induced the accumulation of eosinophils in skin. Mice were injected i.v. with IL-5 [100 pmol/kg in 100 ml of 10 mM PBS/0.1% BSA (pH 7.4)] and 30 minutes later were given a s.c. injection of (a) MIP-1α (0.005-1 pmol/skin site), (b) RANTES (0.05-10 pmol/skin site) or control vehicle [100 µl of HBSS/0.01% BSA (pH 7.4)]. Mice were sacrificed 2 hours after s.c. injections and the accumulation of eosinophils in the dorsal membrane determined. Results represent mean eosinophils/mm^2 ± SEM of groups of 6 mice. One dorsal membrane preparation was excised from each animal and prepared for differential cell counting. Ten fields per preparation were counted for eosinophil infiltration and the mean obtained. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.01$. $*P < 0.05$ and $** P < 0.001$ when compared with control vehicle.


(a)
2
1
0
eosinophils/mm^2 x 10^2
**
*
EC$_{50}$
0.00
0.01
0.10
1.00
MIP-1α pmol/skin site

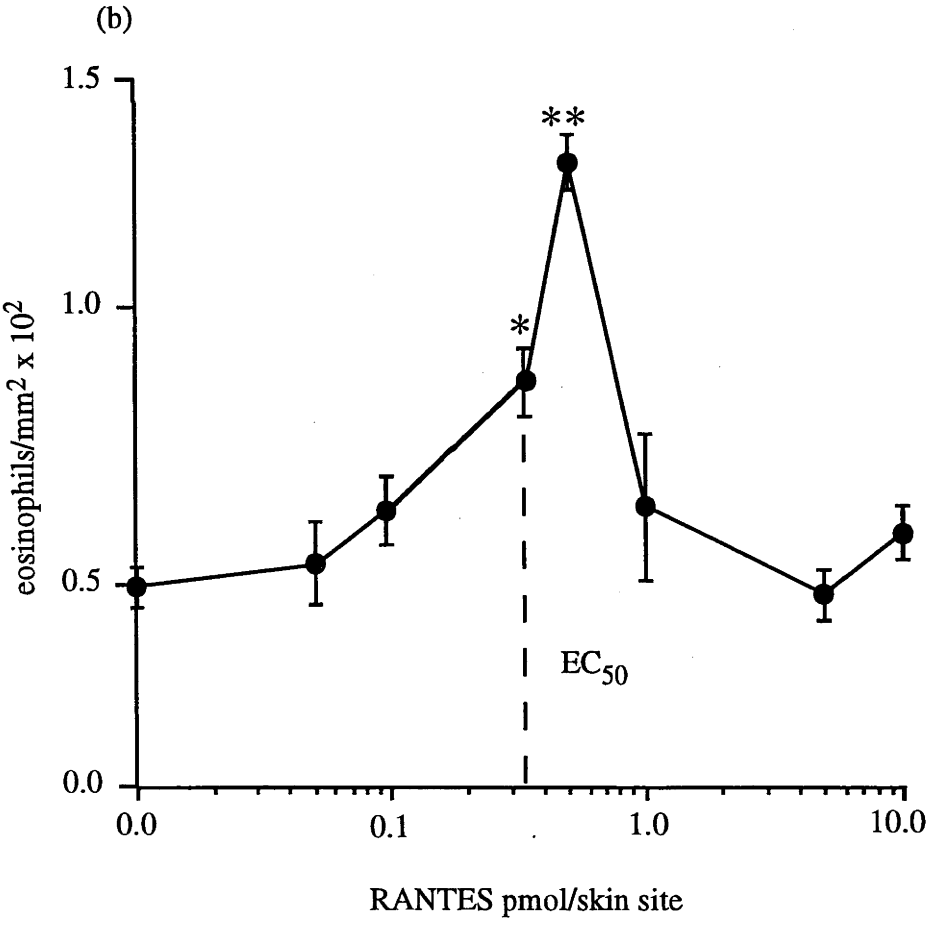
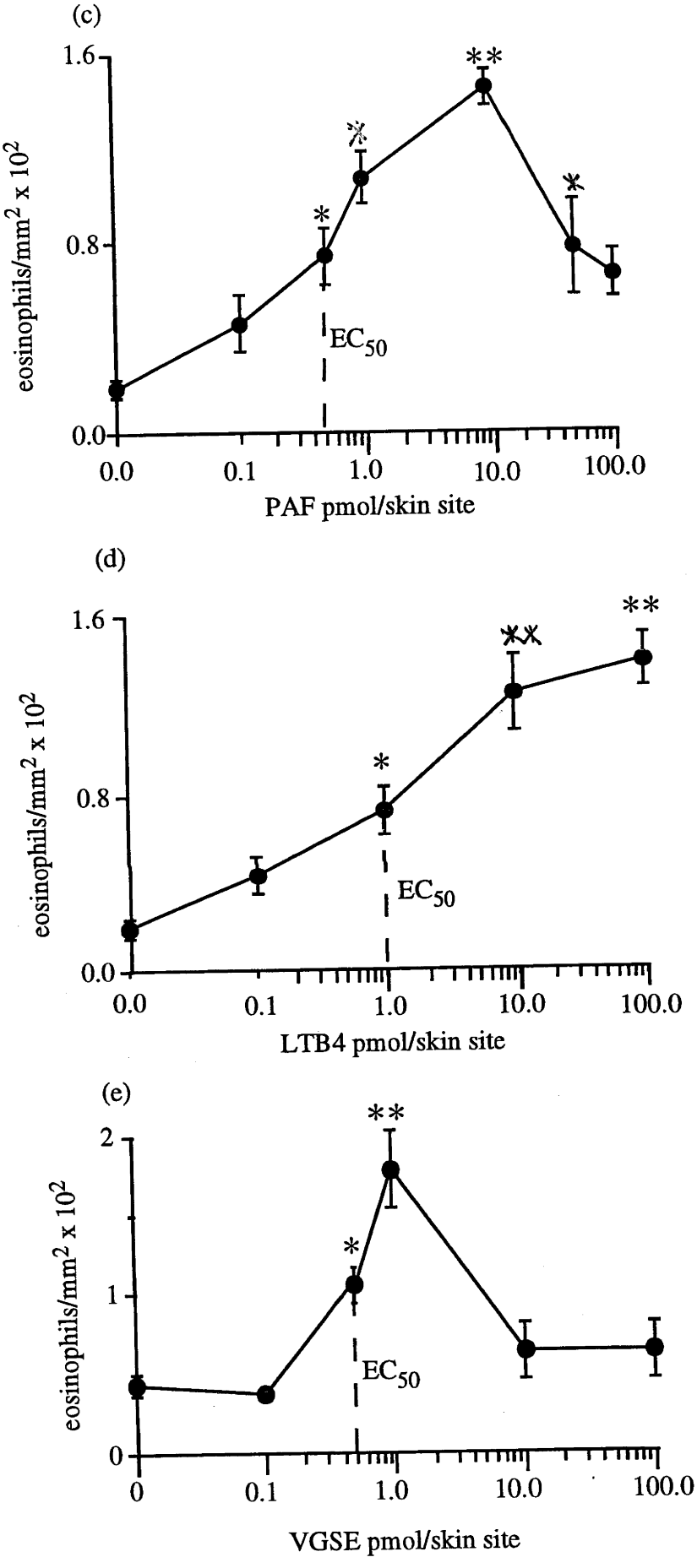

(b)
1.5
1.0
0.5
0.0
eosinophils/mm^2 x 10^2
**
*
EC$_{50}$
0.0
0.1
1.0
10.0
RANTES pmol/skin site

Figure II.6 ***Dose response curves for PAF, LTB_4 and VGSE and determination of half maximally effective concentrations for the accumulation of eosinophils in skin.***

PAF, LTB_4 and VGSE potently induced the accumulation of eosinophils in skin. Mice were injected i.v. with IL-5 [100 pmol/kg in 100 ml of 10 mM PBS/0.1% BSA (pH 7.4)] and 30 minutes later were given a s.c. injection of (a) PAF (0.1-100 pmol/skin site), (b) LTB_4 (0.05-10 pmol/skin site), (c) VGSE (0.1-100 pmol/skin site) or control vehicle [100 µl of HBSS/0.01% BSA (pH 7.4)]. Mice were sacrificed 2 hours after s.c. injections and the accumulation of eosinophils in the dorsal membrane determined. Results represent mean eosinophils/mm^2 ± SEM of groups of 6 mice. One dorsal membrane preparation was excised from each animal and prepared for differential cell counting. Ten fields per preparation were counted for eosinophil infiltration and the mean obtained. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.01$. $^*P < 0.05$ and $^{**}P < 0.001$ when compare with control vehicle.


(c)
1.6
0.8
0.0
eosinophils/mm^2 x 10^2
**
*
*
*
EC$_{50}$
0.0
0.1
1.0
10.0
100.0
PAF pmol/skin site
(d)
1.6
0.8
0.0
eosinophils/mm^2 x 10^2
**
**
*
EC$_{50}$
0.0
0.1
1.0
10.0
100.0
LTB4 pmol/skin site
(e)
2
1
0
eosinophils/mm^2 x 10^2
**
*
EC$_{50}$
0
0.1
1.0
10.0
100.0
VGSE pmol/skin site

The order of potency of these chemoattractants on a molar basis was, MIP-1α (EC_{50} = 0.05 pmol) > RANTES (EC_{50} = 0.25 pmol) > VGSE (EC_{50} = 0.5 pmol) = IL-5 (EC_{50} = 0.5 pmol) = PAF (EC_{50} = 0.5 pmol) > LTB_4 (EC_{50} = 1 pmol) > eotaxin (EC_{50} = 2.5 pmol). The co-administration of eotaxin and IL-5 at their respective EC_{50}, induced the accumulation of eosinophils in the skin at 2 hours to levels greater than that induced by maximally effective concentrations of either cytokine alone or a calculated additive effect determined by combining the levels of eosinophil accumulation by each of these molecules at their respective EC_{50}, less the control value (figure II.7a). The co-injection of IL-5 and other members of the C-C-chemokine family, namely, MIP-1α (figure II.7b) and RANTES (figure II.7c) at their respective EC_{50}, also induced a potent eosinophilia in the skin that was synergistic (figure II.7). As with IL-5 and eotaxin, the accumulation of eosinophils in response to IL-5 and MIP-1α or IL-5 and RANTES was greater than that induced by either of these cytokines alone at maximally effective concentrations or when compared to a calculated additive level of eosinophil accumulation.

To determine whether it was the action of IL-5 or the C-C-chemokine which inferred synergy on the accumulation of eosinophils in skin and whether this synergy could be referred to other non-cytokine eosinophil chemoattractants, IL-5 or eotaxin were co-injected with LTB_4 (figure II.8a), PAF (figure II.8b) or VGSE (figure II.8c), each at their respective EC_{50}. Interesting, the synergistic action of IL-5 on the accumulation of eosinophils induced by either eotaxin, MIP-1α or RANTES, did not extend to the two lipid mediators (figure II.8a and figure II.b) or to VGSE (figure II.8c). Similarly, the lipid mediators did not act synergistically with each other (figure II.9c) or with eotaxin (figures II.9 a and II.9b) to promote the accumulation of eosinophils at sites of administration in the skin.

Figure II.7 ***IL-5 acts synergistically with the C-C-chemokines, eotaxin, MIP-1α and RANTES to promote the accumulation of eosinophils in skin.***

Mice were given an i.v. injection of IL-5 (100 pmol/kg) and 1 hour later injected s.c. with IL-5 and either eotaxin, MIP-1α or RANTES, each at EC_{50}. Mice were sacrificed 2 hours post s.c. injection and the accumulation of eosinophils in the dorsal membrane determined. IL-5 (0.5 pmol/skin site) in combination with (a) eotaxin (2.5 pmol/skin site), (b) MIP-1α (0.0625 pmol/skin site) or (c) RANTES (0.25 pmol/skin site) induced the accumulation of eosinophils in the skin at levels greater than that induced by either of the molecules at maximal effective concentration or what would be expected by the additive effect of both molecules in isolation at EC_{50} less control levels. Results represent mean eosinophils/mm^2 ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) * $P < 0.02$ compared to eotaxin or IL-5 alone at EC_{100}, (b) * $P < 0.02$ compared to IL-5 or MIP-1α alone at EC_{100} and (c) $P < 0.02$ compared to IL-5 or RANTES alone at EC_{100}.

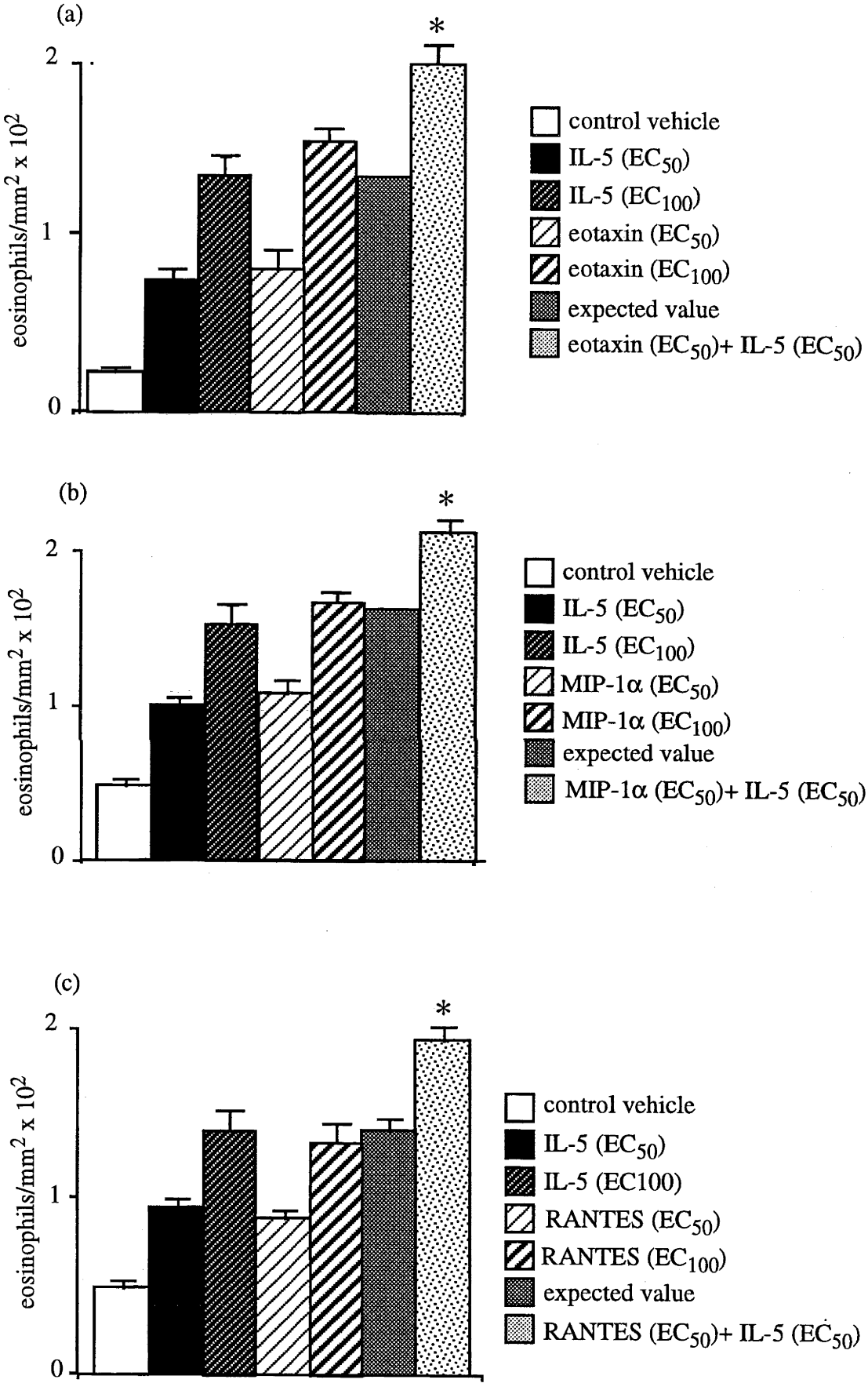

(a)
eosinophils/mm^2 x 10^2
2
1
0
*
control vehicle
IL-5 (EC$_{50}$)
IL-5 (EC$_{100}$)
eotaxin (EC$_{50}$)
eotaxin (EC$_{100}$)
expected value
eotaxin (EC$_{50}$)+ IL-5 (EC$_{50}$)
(b)
eosinophils/mm^2 x 10^2
2
1
0
*
control vehicle
IL-5 (EC$_{50}$)
IL-5 (EC$_{100}$)
MIP-1α (EC$_{50}$)
MIP-1α (EC$_{100}$)
expected value
MIP-1α (EC$_{50}$)+ IL-5 (EC$_{50}$)
(c)
eosinophils/mm^2 x 10^2
2
1
0
*
control vehicle
IL-5 (EC$_{50}$)
IL-5 (EC100)
RANTES (EC$_{50}$)
RANTES (EC$_{100}$)
expected value
RANTES (EC$_{50}$)+ IL-5 (EC$_{50}$)

Figure II.8 ***IL-5 does not act synergistically with PAF, LTB_4 or VGSE to promote the accumulation of eosinophils in skin.***

Mice were given an i.v. injection of IL-5 (100 pmol/kg) and 1 hour later injected s.c. with EC_{50} of IL-5 (0.5 pmol/skin site) and either PAF (0.5 pmol/skin site), LTB_4 (1.0 pmol/skin site) or VGSE (0.5 pmol/skin site). Mice were sacrificed 2 hours post s.c. injection and the accumulation of eosinophils in dorsal skin membranes analysed. IL-5 in combination with (a) PAF, (b) LTB_4 or (c) VGSE induced the accumulation of eosinophils in the skin at sites of co-administration at levels that was equivalent to that induced by either molecule at EC_{100} or when compared with a level that was calculated by the addition of levels of eosinophil accumulation induced by both molecules alone at EC_{50} less the control level. Results represent mean eosinophils/mm^2 ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. No significant differences were detected between IL-5 at EC_{50} in combination with (a) PAF, (b) LTB_4 or (c) VGSE at EC_{50} and IL-5, (a) PAF, (b) LTB_4 or (c) VGSE alone at EC_{100}.

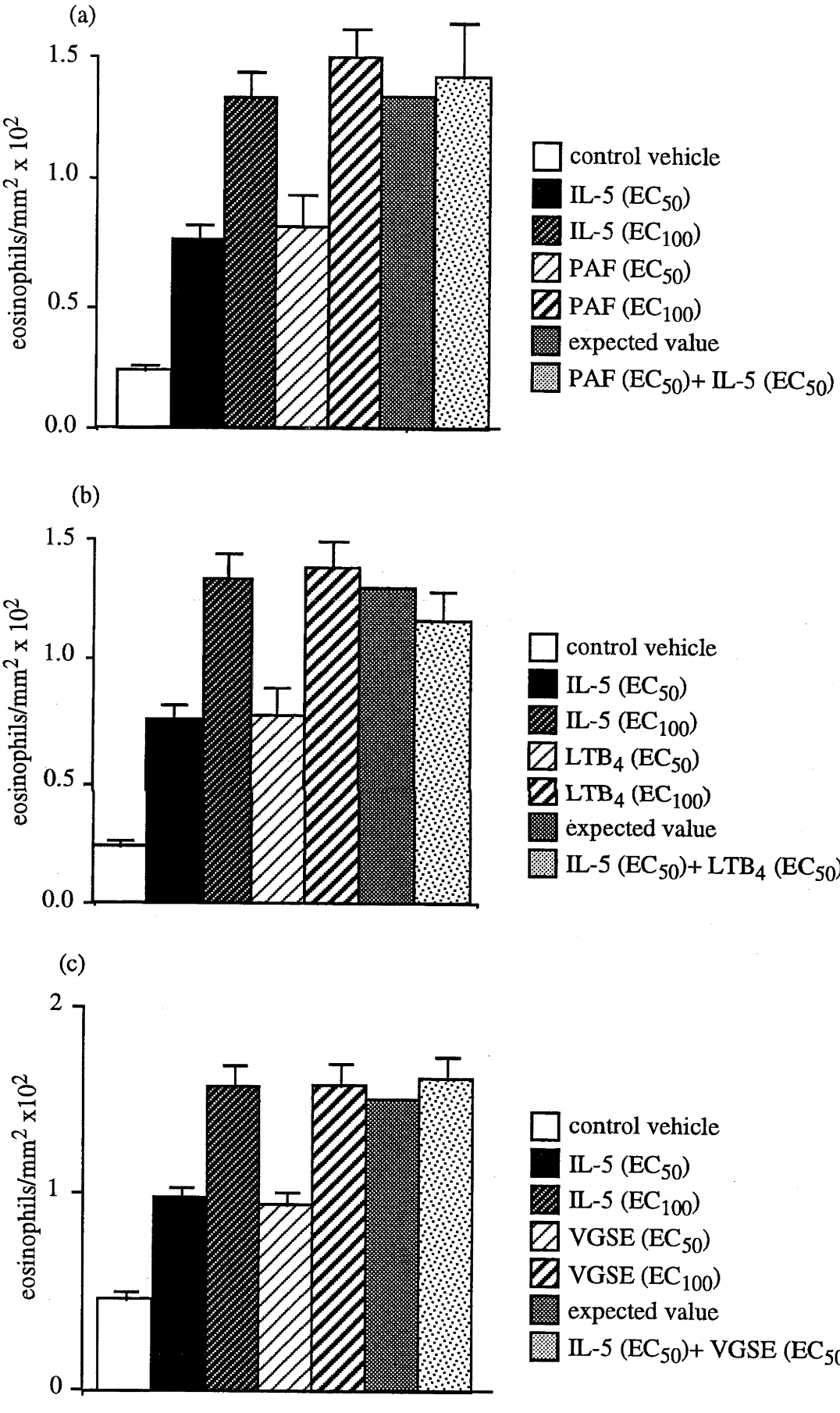
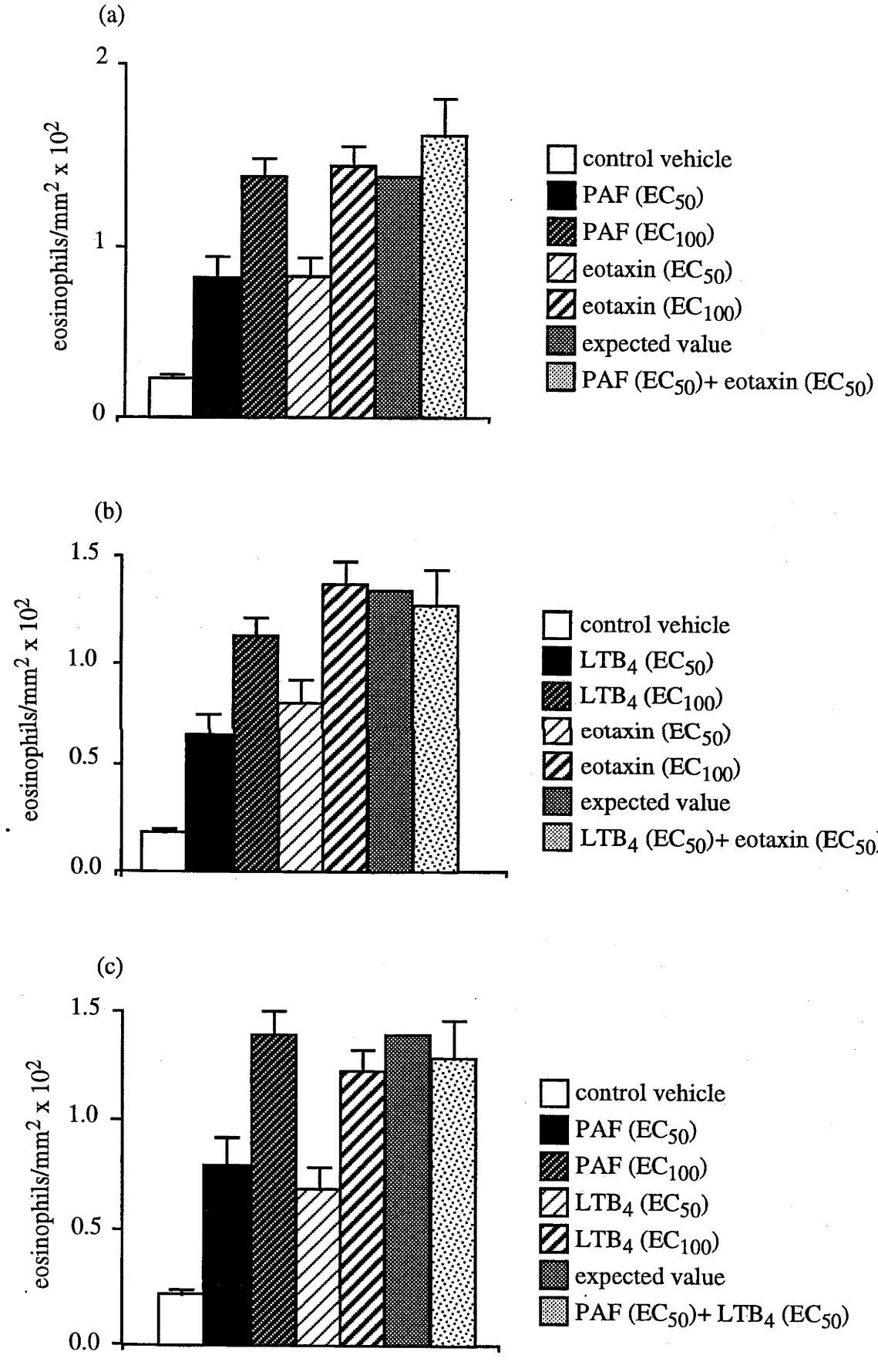

(a)
1.5
1.0
0.5
0.0
eosinophils/mm^2 x 10^2
control vehicle
IL-5 (EC$_{50}$)
IL-5 (EC$_{100}$)
PAF (EC$_{50}$)
PAF (EC$_{100}$)
expected value
PAF (EC$_{50}$)+ IL-5 (EC$_{50}$)
(b)
1.5
1.0
0.5
0.0
eosinophils/mm^2 x 10^2
control vehicle
IL-5 (EC$_{50}$)
IL-5 (EC$_{100}$)
LTB$_4$ (EC$_{50}$)
LTB$_4$ (EC$_{100}$)
expected value
IL-5 (EC$_{50}$)+ LTB$_4$ (EC$_{50}$)
(c)
2
1
0
eosinophils/mm^2 x10^2
control vehicle
IL-5 (EC$_{50}$)
IL-5 (EC$_{100}$)
VGSE (EC$_{50}$)
VGSE (EC$_{100}$)
expected value
IL-5 (EC$_{50}$)+ VGSE (EC$_{50}$)

Figure II.9 ***Eotaxin and PAF or LTB_4 or PAF and LTB_4 do not act synergistically to promote the accumulation of eosinophils in skin.***

Mice were given an i.v. injection of IL-5 (100 pmol/kg) and then 1 hour later were injected s.c. with eotaxin (2.5 pmol/skin site) and either PAF (0.5 pmol/skin site) or LTB_4 (1.0 pmol/skin site) each at EC_{50}. Another group were injected i.v. with IL-5 and then one hour later were injected s.c. with PAF (0.5 pmol/skin site) and LTB_4 (1.0 pmol/skin site) each at EC_{50}. Mice were sacrificed 2 hours post s.c. injection and the accumulation of eosinophils in the dorsal skin membrane determined. (a) Eotaxin in combination with PAF or (b) eotaxin in combination with LTB_4 induced the accumulation of eosinophils at sites of administration in the skin at levels that were equivalent to that induced by the EC_{100} value of (a) eotaxin or PAF or (b) LTB_4 and the expected levels that were calculated by the addition of the levels of eosinophil accumulation induced by each molecule at EC_{50} less the control level. (c) The co-administration of PAF (0.5 pmol/skin site) and LTB_4 (1.0 pmol/skin site) induced the accumulation of eosinophils in the skin to levels that were equivalent to that induce by EC_{100} of these molecules alone and the calculated expected levels. Results represent mean eosinophils/mm^2 ± SEM of groups of 6 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. No significant differences were found between groups that received co-injections of EC_{50} of chemoattractants and the groups that received these respective molecules alone at maximally effective concentrations.


(a)
2
1
0
eosinophils/mm^2 x 10^2
control vehicle
PAF (EC$_{50}$)
PAF (EC$_{100}$)
eotaxin (EC$_{50}$)
eotaxin (EC$_{100}$)
expected value
PAF (EC$_{50}$)+ eotaxin (EC$_{50}$)
(b)
1.5
1.0
0.5
0.0
eosinophils/mm^2 x 10^2
control vehicle
LTB$_4$ (EC$_{50}$)
LTB$_4$ (EC$_{100}$)
eotaxin (EC$_{50}$)
eotaxin (EC$_{100}$)
expected value
LTB$_4$ (EC$_{50}$)+ eotaxin (EC$_{50}$)
(c)
1.5
1.0
0.5
0.0
eosinophils/mm^2 x 10^2
control vehicle
PAF (EC$_{50}$)
PAF (EC$_{100}$)
LTB$_4$ (EC$_{50}$)
LTB$_4$ (EC$_{100}$)
expected value
PAF (EC$_{50}$)+ LTB$_4$ (EC$_{50}$)

II.4 DISCUSSION.

The selectivity and kinetics of tissue expression of eotaxin, suggest a central role for this chemokine in the early phases of eosinophil homing during allergic inflammation (Jose *et al.,* 1994a; Rothenberg *et al.,* 1995b; Collins *et al.,* 1995; Gonzalo *et al.,* 1996b). Both eotaxin and IL-5 induced a blood eosinophilia when administered i.v. (figure II.1). The inability of i.v. eotaxin to mobilise bone marrow eosinophils, in contrast to IL-5, suggests the existence of two distinct eosinophil pools which can be mobilised in response to these cytokines. IL-5 appears to mobilise a bone marrow eosinophil pool, while eotaxin may sequester eosinophils that are residing in tissues into the circulation (figure II.2). During allergic cutaneous inflammation two phases (peaks at 6 and 24 hours) of tissue eosinophilia are observed (Iwamoto *et al.,* 1992). Previous reports indicate that the first phase of eosinophilia can occur independently of IL-5, while the second phase is significantly reduced in the presence of anti-IL-5 mAb (Iwamoto *et al.,* 1992). The expression of eotaxin occurs early in the inflammatory response and can be induced in cultured endothelial cells, alveolar epithelial cells and T-cell clones (Th_1 and Th_2 clones) by cytokines (Rothenberg *et al.,* 1995a; Gonzalo *et al.,* 1996b). Taken together, these results suggest that eotaxin secreted from inflammatory cells or tissue sites in the early phases of the inflammatory response may be able to initiate and supplement tissue eosinophilia by sequestering, into the circulation, eosinophils which are migrating through non-inflamed tissues. This mechanism would utilise the eosinophil pool already participating in immune surveillance and provide a mechanism for an immediate response to allergic inflammation or parasite infection in tissues. Eosinophilic inflammation could then be amplified and sustained over the course of the inflammatory response by eosinophils mobilised by IL-5 from the bone marrow. The observation that i.v. co-administration of both IL-5 and eotaxin causes blood eosinophilia to a level that is greater than that induced by either of these cytokines alone further suggests that these two cytokines may act in synergy during allergy to enhance the circulating eosinophil pool. Recently, IL-5 was also shown to mobilise eosinophils from the bone marrow and promote eosinophilic responses to s.c. eotaxin, supporting this concept (Collins *et al.,* 1995).

Eotaxin is a potent eosinophil chemoattractant *in vivo* (Jose *et al.*, 1994a). Eotaxin potently induced the accumulation of eosinophils in the skin at sites of administration at doses ranging from 0.1-10 pmol/site and had an inhibitory effect at higher doses (figure II.3). The narrow range in which eotaxin stimulates eosinophilia suggests that chemotactic signals for the selective migration of a cell type are discretely regulated at the site of inflammation. The rapid action of eotaxin on eosinophil trafficking in the skin (2 hours) also suggests that this molecule promotes the accumulation of eosinophils directly by acting as a chemotactic agent, although it is possible that this chemokine may also

stimulate the release of other preformed chemoattractants from cells at the site of administration. Following the completion of experiments in this Chapter, eotaxin was shown to regulate eosinophil recruitment to the allergic inflamed lungs of mice (Rothenberg *et al.*, 1997). Eosinophil recruitment was significantly reduced during the early stages (18 hours) of allergic inflammation. Interestingly, no difference in the accumulation of eosinophils during later stages (48 hours) of the inflammatory reaction of the allergic response was reported. These reports support the hypothesis that eotaxin is an important molecule for the rapid recruitment of eosinophils to sites of allergic inflammation.

Similar to eotaxin, IL-5 was found to potently induce the accumulation of eosinophils in the skin; suggesting that IL-5 secreted in tissue compartments can act as a chemoattractant for eosinophils as well as enhance other chemotactic signals for the accumulation of eosinophils *in vivo* (Wang *et al.,* 1989; Iwama *et al.,* 1993; Coeffier *et al.,* 1994; Collins *et al.,* 1995). Notably, it was demonstrated that eotaxin and IL-5 act cooperatively to enhance the accumulation of eosinophils in tissues (figure II.4).

Intravenous IL-5 was also shown to enhance the accumulation of eosinophils in tissues in response to eotaxin. Interestingly, despite eotaxin's potent induction of blood eosinophilia, i.v. eotaxin did not enhance the accumulation of eosinophils in the skin in response to itself or IL-5 (figure II.4b). This indicates that elevated blood levels of eosinophils alone are not sufficient to enhance eosinophil recruitment into tissues in response to chemoattractants. The ability of i.v. IL-5 to enhance eosinophil recruitment into tissues may involve the priming of this cell in the circulation, promoting migration in response to s.c. eotaxin (or IL-5), in addition to elevating the circulating eosinophil pool. Thus, IL-5 may have more than one functional role in the promotion of eosinophil recruitment. IL-5 and eotaxin cooperatively induced blood eosinophilia and enhanced the accumulation of eosinophils in tissues in response s.c. eotaxin or IL-5 to a greater degree than that observed by treatment with i.v. IL-5 alone. These observations further demonstrate the cooperative nature of the interaction between eotaxin and IL-5 for eosinophil homing.

All of the chemoattractants tested (eotaxin, RANTES, MIP-1α, LTB_4, PAF and the ECF-a tetra peptide, VGSE) potently induced the accumulation of eosinophils in the skin. Interestingly, in these experiments, VGSE and PAF were equipotent. This observation is in contrast to previous reports by Wardlaw *et al.*, (1986) which showed that PAF was more potent than VGSE at inducing human eosinophil chemotaxis *in vitro*. Species differences may account for the inconsistencies between *in vitro* studies and our *in vivo* experiments. As shown above, the cooperative action of IL-5 and eotaxin for the induction of eosinophilia was synergistic (figure II.7a). IL-5 also acted in synergy with

MIP-1α (figure II.7b) and RANTES (figure II.7c) to promote the accumulation of eosinophils *in vivo* and thus, this phenomena may be an effect of IL-5 on C-C-chemokine signalling. Interestingly, IL-5 did not act in synergy with VGSE or lipid mediators (LTB_4 and PAF). Furthermore, eotaxin itself did not exhibit any synergistic activity with other eosinophil chemoattractants (other than IL-5), which further indicates that it is IL-5 that contributes to this synergistic phenomenon.

IL-5 may selectively prime eosinophils to C-C chemokine signals. Eotaxin and RANTES are agonists for CCR3 which is expressed by eosinophils (Daugherty *et al.*, 1996). Although the agonistic effect of MIP-1α on the human eosinophil CCR3 is controversial (Combadierie *et al.*, 1995a, Daughtery *et al.*, 1996), MIP-1α, RANTES and eotaxin may all signal through CCR3. It has recently been shown that the action of RANTES and eotaxin on the chemotaxis of human eosinophils is primarily (95%) mediated through this chemokine receptor (Heath *et al.*, 1997). Collectively, these results and those of others strongly suggest that IL-5 may promote eosinophil trafficking in response to MIP-1α, RANTES and eotaxin by potentiating CCR3-activated intracellular signalling pathways.

During the course of these experiments, Okada *et al.*, (1997) demonstrated that IL-5 may act synergistically with eosinophil chemoattractants such as eotaxin and PAF to promote eosinophil transmigration *in vitro* and these effects were dependent on the VLA-4 and CD18 adhesion pathways. Results in this Chapter showed that the i.v. administration of IL-5 amplified the accumulation of eosinophils in the skin in response to eotaxin. Although peripheral blood eosinophil levels are elevated by i.v. IL-5 administration, this cytokine may also amplify the recruitment of eosinophils into tissues, in part, by promoting transmigration events. Interestingly, in these experiments, no synergy was observed between IL-5 and PAF on the accumulation of eosinophils. These results indicate that these two molecules did not act synergistically, to direct chemotaxis, unlike IL-5 and the C-C chemokines (eotaxin, MIP-1α and RANTES).

The synergistic action of IL-5 with the eosinophil active C-C chemokines allows these molecules to efficiently and potently promote the accumulation of eosinophils in tissues at concentrations which would normally be suboptimal for these molecules individually. Interestingly, this synergy appears to occur between molecules that selectively induce eosinophil migration (IL-5 and the eosinophil active C-C chemokines: eotaxin, MIP-1α and RANTES), but not between IL-5 and lipid mediators (LTB_4 and PAF) that also act on neutrophils. This suggests that this synergistic phenomenon is part of an eosinophil selective trafficking pathway which may provide a mechanism for the selective accumulation of eosinophil at sites of allergic inflammation. Eotaxin, MIP-1α and RANTES are expressed in tissues of sensitised mice following antigen challenge (Gonzalo *et al.*, 1996a, 1996b; Alam *et al.*, 1996). Thus, these chemokines may act in

synergy with IL-5 to provide a potent and selective mechanism for the recruitment of eosinophils into inflamed tissues during allergic reactions.

It would be interesting to further elucidate the synergistic mechanism operating between IL-5 and the C-C-chemokines, RANTES, MIP-1α and eotaxin. Such studies could be directed at examining the action of IL-5 of the CCR3 signalling pathway since all of these chemokines may signal through this receptor. Monitoring parameters such as cell motility with transient changes in intracellular-signalling molecules and free calcium may further our understanding of the processes that underlie the selective trafficking of eosinophils into tissues and may identify targets for the treatment of allergic diseases.

CHAPTER III

THE RELATIONSHIP BETWEEN INTERLEUKIN-5 AND EOTAXIN IN REGULATING PULMONARY EOSINOPHILIA AND THEIR ROLE IN AIRWAYS DYSFUNCTION IN MICE

III.1 INTRODUCTION.

Eosinophilic inflammation of the airways is a major characteristic of asthma and is believed to underlie the pathophysiological changes to the lung. Of the numerous inflammatory cytokines implicated in eosinophil trafficking during allergy it has become increasingly obvious that both IL-5 and eotaxin play central roles (Jose *et al.,* 1994a; Rothenberg *et al.,* 1995b; Baggiolini, 1996; Gonzalo *et al.,* 1996b; Foster *et al.,* 1996).

Unlike guinea pigs, mice do not develop pulmonary eosinophilia following the instillation of recombinant eotaxin into the lungs (Rothenberg *et al.,* 1996). However, the instillation of this chemokine into the lungs of IL-5 transgenic mice that over express this cytokine, potently induces pulmonary eosinophilia in a dose dependent manner (Rothenberg *et al.,* 1996). This indicates that IL-5 acts with eotaxin to regulate eosinophil trafficking in mice. The ability of IL-5 to enhance eotaxin-induced pulmonary eosinophilia may be due to the increased levels of circulating eosinophils in IL-5 transgenic mice (Rothenberg *et al.*, 1996). In Chapter II and other investigations (Collins *et al.,* 1995), it has been shown that IL-5 cooperatively enhances eosinophilic responses to eotaxin. The action of IL-5 in these processes may be two fold; IL-5, acting in synergism with eotaxin may promote eosinophil migration into tissues, in addition to elevating the circulating eosinophil pool.

In transgenic mice, where IL-5 was specifically over expressed in the airway epithelium, both pulmonary eosinophilia and airways hyperreactivity developed (Lee *et al.,* 1997). This suggests a central role for IL-5 in the pathogenesis of asthma. In contrast, although the instillation of recombinant IL-5 into airways of guinea pigs induced a pulmonary eosinophilia, it did not promote the development of airways hyperreactivity (Lilly *et al.*, 1996). These apparently conflicting roles of IL-5 in the development of airway hyperreactivity may be due to species difference and/or the duration of IL-5 exposure in these two studies.

IL-5 has a multitude of actions on eosinophil function (eg. haempoiesis, priming, migration, degranulation and survival [refer to introduction]). Eotaxin, although primarily characterised as an eosinophil chemoattractant (Rothenberg *et al.,* 1996), also potently induces respiratory bursts in this cell (Elsner *et al.*, 1996). The aims of the experiments in this Chapter were to determine the ability of, and the relationship between, IL-5 and eotaxin to regulate eosinophil recruitment to pulmonary tissues. Furthermore, since the role of eotaxin and IL-5 in eosinophil activation and the effect of these two molecules on pulmonary function is unknown, the effect of these two cytokines on airways structure and function in the presence and absence of antigen processing in the lung was also examined.

Due the reputed poor induction of pulmonary eosinophilia by the instillation of eotaxin in the lungs of mice (Rothenberg *et al.*, 1996), recombinant vaccinia viruses encoding the eotaxin or IL-5 genes were constructed (Ramsay and Kohonen-Corish, 1993) to determine the effects of transient expression of these two cytokines on pulmonary eosinophilia and airways function.

In contrast to transgenic mice, the vaccinia virus system for the expression of cytokines provides a unique way of studying the effects of localised transient cytokine expression in the lung. Another advantage of the vaccinia virus system over the transgenic manipulation of animals is that it avoids exposing animals to abnormally high levels of molecules during development, which can adversely alter physiological responses. By using the recombinant eotaxin and IL-5 -encoding vaccinia viruses, the roles of each of these molecules in isolation and in combination, upon the induction of pulmonary eosinophilia was examined. Furthermore, this system allowed for the study of the effect of eosinophils on airways function in the absence of other inflammatory cells associated with Th_2 responses.

III.2 MATERIALS AND METHODS.

III.2.1 Cloning of VV-HA-eotaxin.

Refer to figure III.1 for a graphic representation of this process.

III.2.1.1 *Cloning of eotaxin cDNA from MR#310 into the vaccinia virus vector pPS 7.5A.*

Murine eotaxin cDNA (21-328 bp of full length murine eotaxin which encodes the mature protein), cloned into plasmid PCRII (Invitrogen, Carlsbad, CA., USA.) with 5' *Hind* III and 3' *Eco* R1 restriction enzyme sites, was a gift from Professor Mark Rothenberg (Childrens Hospital Medical Centre, Cincinnati, OH., USA.) and was denoted MR#310.

Due to the incompatibility of common restriction sites between MR#310 and the multiple cloning site of the vaccinia virus vector, pPS 7.5a (Coupar *et al.,* 1988), the eotaxin cDNA was excised (restriction enzyme digested) from MR#310 and blunt-end ligated into pPS 7.5a. Briefly, pPS 7.5a was digested with *Hind* III and *Bam* HI and the resultant 5' overhangs end-filled to form blunt-end termini using Klenow polymerase (Promega, Madison, WI., USA.). Five prime blunt-end termini were dephosphorylated using calf intestinal alkaline phosphatase to prevent self-ligation. Eotaxin cDNA was excised from MR#310 using *Hind* III (5') and *Eco* R1 (3'). The resultant 5' overhangs were end filled to form blunt end termini using Klenow polymerase (Promega, Madison, WI., USA.). Eotaxin cDNA was ligated to the linearized pPS 7.5a using ligase (Sambrook *et al.*, 1989) and the orientation of the inserted eotaxin cDNA was determined by restriction enzyme digest with *Bgl* II and *Eco* R1. The resultant plasmid containing eotaxin cDNA was named pAM#1.

III.2.1.2 *Maintenance of HuTK$^-$143B cells.*

Human thymidine kinase negative 143B cells (143B cells) were grown in F15 Complete [F15 supplemented with 5% heat inactivated foetal calf serum (FCS), 2 mM L-glutamine and 10 mM HEPES buffer] at 37°C/5% $C0_2$. Cells were passaged upon confluence by aspirating the media off, washing the monolayer with PBS and incubating the cells at 37°C with enough 0.25% trypsin/0.02% EDTA in HBSS to cover the monolayer until the cells detached. Ten millilitres of F15 Complete was added to arrest trypsinization and new flasks seeded with cells at 4 x 10^4 cells/cm^2/0.20-0.25 mls F15 Complete.

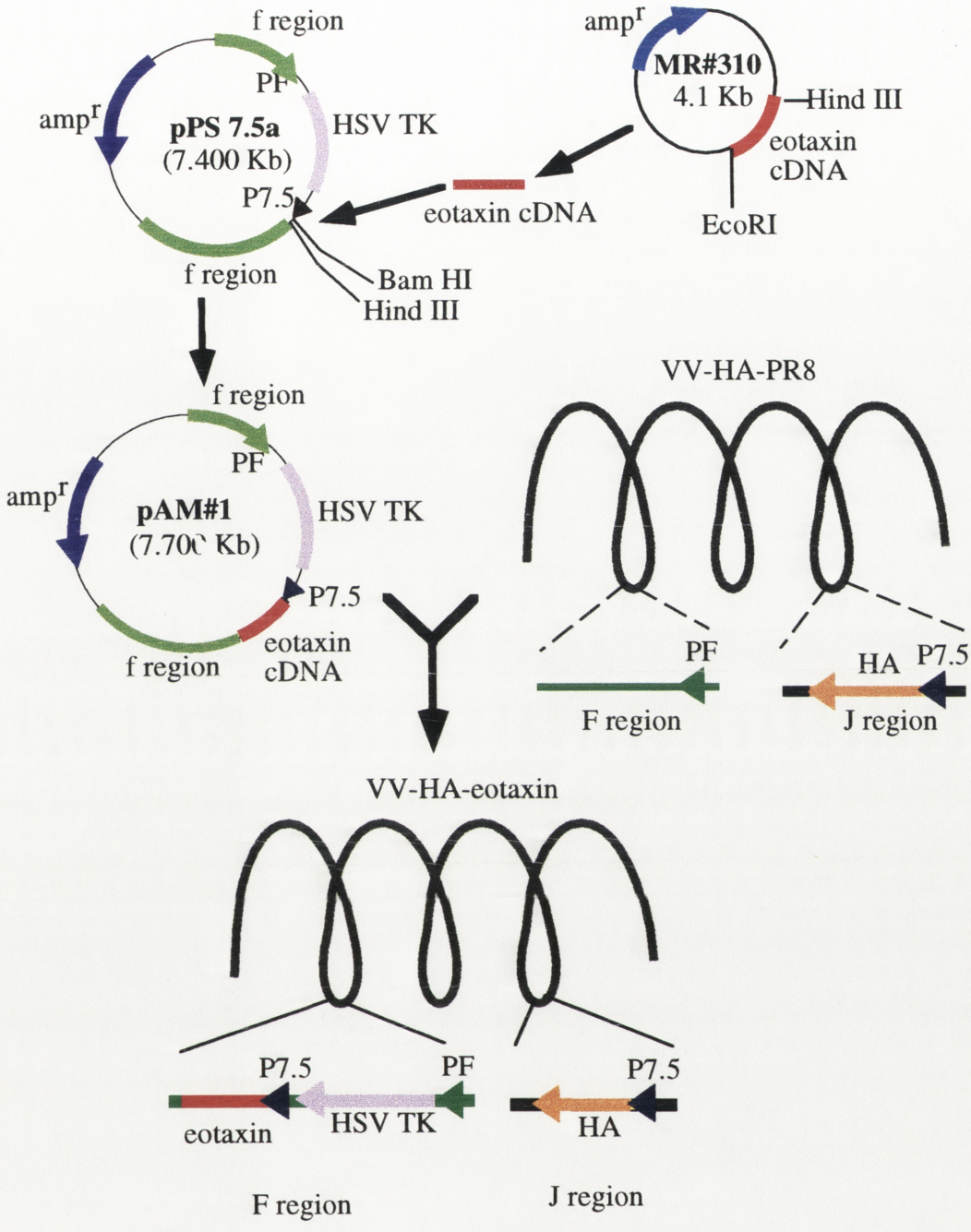


Figure III.1 ***Cloning of VV-HA-eotaxin.***

Eotaxin cDNA was excised from MR#310 and ligated into pPS7.5a (Coupar *et al.*, 1988). The resulting vaccinia virus vector containing eotaxin cDNA was called pAM#1 and was transfected into 143B cells with VV-HA-PR8 (Coupar *et al.*, 1988). Homologous recombination of the F regions of VV-HA-PR8 with pAM#1 formed the recombinant VV-HA-eotaxin.

III.2.1.3 *Transfection and viral recombination.*

VV-HA-eotaxin was constructed from VV-HA-PR8 (Coupar *et al.*, 1988) which carries the hemagglutinin (HA) gene of the influenza virus A/PR/8/34 in the *Hind* III J region and the thymidine kinase (TK) gene of the herpes simplex virus (HSV) in the *Hind* III F region. Recombinant virus was purified using marker rescue of the TK gene (Coupar *et al.*, 1988) and methotrexate selection (Panicali and Paoletti, 1982). Briefly, near confluent 143B cells in a 25 cm^2 tissue culture flask were infected with VV-HA-PR8 at a multiplicity of infection (M.O.I.) of 0.05. Sixty minutes after viral infection the cells were transfected with 20 µg pAM#1 using DOTAP transfection reagent (Boehringer Mannheim, Castle Hill, NSW., Australia) according to the manufacturer's instructions. After overnight incubation at 37°C/5% CO_2, fresh F15 Complete, supplemented with MTAGG solution (3 mM methotrexate, 15 mM thymidine, 50 mM adenosine, 50 mM guanosine and 100 mM glycine) was added and the cells incubated for a further two days.

Cells were harvested using a cell scraper and pelleted by centrifugation (400 x g for 5 minutes at 4°C). The cell pellet was resuspended in 1 ml of gelatine saline/20 mM HEPES (GSH). Virus was freed from the cells by trypsinization (100 mg/ml trypsin, 37°C for 30 minutes) and was amplified by infecting 143B cells in a 25 cm^2 tissue culture flask seeded with 1.5 x 10^6 cells the previous day. After 2 days incubation at 37°C/5%$C0_2$ under MTAGG selection, the virus infected cells were harvested using a cell scraper and resuspended in 1 ml of GSH.

III.2.1.4 *Primary plaque purification using TK^{+ve} selection.*

Logarithmic dilutions (100 µl) of trypsinised (see section III.2.1.3) viral stock (10^{-1} to 10^{-6}) were added drop-wise to drained 143B cells in a 6 well tissue culture plate that had been seeded with 8 x 10^5 cells/well the previous day. After incubation at 37°C/5% CO_2 for 60 minutes, 2.5 mls of F15 Complete/MTAGG selection media was added and the cells were incubated for a further 2 days. The media was then removed and 2.5 mls of neutral red (0.01% in PBS) added to each well and the virus plaques counted. Individual plaques were collected using a micro pipette and were added to semi drained 143B cells in a 24 well tissue culture plate that had been seeded the previous day at 1.67 x 10^5 cells/well. Following 2 days of incubation at 37°C/5% CO_2 under MTAGG selection, each well was scored for cytopathic effect and the drained cells lysed (using 20 µl of sterile ddH_20) to liberate virus. The viral mix was then tested for the presence of the eotaxin cDNA using dot blot hybridisation (see section III.2.1.5).

The 24 well tissue culture plate was freeze/thawed 3 times and 10 µl from each well was spotted onto a nitrocellulose membrane and air dried. pAM#1 and VV-HA-PR8 DNA

served as positive and negative controls respectively. The nitrocellulose membrane was soaked sequentially in 0.5 M NaOH, 1 M Tris (pH 7.5) and 0.5 M Tris/1.5 M NaCl (pH 7.5) for 5 minutes each, then blotted dry, hot baked at 80°C for 2 hours under vacuum, sealed in plastic and stored at 4°C awaiting radio probing.

III.2.1.5 *Checking primary plaque purifications for the presence of recombinant virus containing the eotaxin cDNA.*

A radioactively labelled eotaxin probe was synthesised from 40 ng of purified eotaxin cDNA using the "Ready To Go" DNA labelling Kit (-dCTP) (Pharmacia Biotec, Uppsala, Sweden), according to the manufacturer's instructions. Unincorporated radio nucleotide was removed using a Sepharose G-50 fine DNA grade Nick column (Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer's instructions.

Nitrocellulose membranes that were dotted with viral DNA were pretreated with a prehybridization mix containing 5 x SSPE [0.18 M NaCl/10 mM NaH_2PO4/1 mM EDTA (pH 7.7)], 0.1% sodium dodoecyl sulphate (SDS), 0.2% Blotto [4% skim milk powder (w/v)/0.02% NaN_3 (w/v)] and 200 µg/ml denatured salmon sperm DNA, at 68°C for 4 hours with shaking. The membranes were then placed into a clean plastic bag containing a hybridization mix [5 x SSPE, 0.1% SDS, 0.2% Blotto and 200 µg/ml salmon sperm DNA and 40 ng of (α–^{32}P) dCTP labelled eotaxin probe (previously denatured at 100°C for 10 minutes)] and were incubated on a roller at 68°C overnight. After washing for 1 minute with 2 x SSC (0.15 M NaCl/1.5 mM trisodium citrate)/0.1% SDS, followed by 30 minutes with 2 x SSC/0.1% SDS, 2 x 15 minutes with 0.5 x SSC /0.1% SDS, 2 x 15 minutes with 0.1 x SSC/0.1% SDS and 2 x 15 minutes with 15 mM NaCl/1.5 mM trisodium citrate/0.1% SDS at 68°C, the membrane was heat sealed in plastic and exposed to Kodak XAR-5 X-ray film with intensifying screen overnight at -70°C. The film was developed using an automatic developer.

Virus underwent two more rounds of plaque purification using MTAGG for TK^+ selection and after the final plaque purification round, virus was checked again for the presence of the eotaxin cDNA using dot blot hybridization as described above.

III.2.1.6 *Checking for parent virus contamination after final round plaque purification using polymerase chain reaction.*

III.2.1.6.1 *Preparation of viral DNA.*

The cell particulate from each well (5 µl) of the 24 well plate from the final round of plaque purification was incubated with proteinase K (0.4 mg/ml) for 60 minutes at 56°C

in 200 µl of 10 mM Tris-HCl (pH 7.6)/50 mM 2-mercaptoethanol/100 mM NaCl/20 mM EDTA/1% sarkosyl (w/v) and 26% sucrose (w/v). After phenol/chloroform extraction, the DNA was ethanol precipitated and resuspended in 30 µl TE buffer containing 20 µg/ml RNase and then stored at 4°C.

III.2.1.6.2 *Polymerase chain reaction amplification of recombination site.*

Viral DNA (5 µl) was added to a 20 µl reaction mix containing 5 pmol of each of FA (forward primer) and FB (reverse primer), 1 x AmpliTaq buffer (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ., USA.), 1 unit of AmpliTaq DNA polymerase (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ., USA.) and 25 µM each of dGTP, dCTP, dATP and dTTP (Promega, Madison, WI., USA.). The PCR primers, FA (5' GTTTAATATGACGCTCG 3') and FB (5' GCGTCACAGAATCTACC 3') that were homologous to the 5' and 3' (of the opposite strand) of the *Hin*d III and *Bam* HI (insert recombination site) sites of the F region, respectively, were provided by Dr D. Boyle, CSIRO Animal Health Laboratory, Australia. The PCR was run using an FTS-1 Thermal Sequencer (Corbett Research, Sydney, Australia) under the following conditions. Ninety four degrees Centigrade for 3 minutes followed by 40 cycles of 3 seconds at 96°C (denaturation), 3 seconds at 50°C (annealing) and 2 minutes at 72°C (extension) followed by a further 7 minutes at 72°C. Ten microlitres of each PCR product was analysed by polyacrylamide gel electrophoresis (PAGE, see section III.2.1.6.2.1). As calculated, the non-homologous recombinant viruses showed a single band approximately 55 bases in length, while homologous recombinants showed a band at approximately 2.4 kilo bases.

III.2.1.6.2.1 *Analysis of polymerase chain reaction products by non-denaturing polyacrylamide gel electrophoresis.*

Electrophoresis was performed through 3.75% stacking/18% resolving non-denaturing polyacrylamide gels (Sambrook *et al.,* 1989) using a Mini-Protean II cell (BioRad, Hercules, CA., USA.) at 200V for 35 minutes. Samples were diluted 1:1 with 2 x sample loading buffer [10 mM Tris HCl (pH 8.0); 20 mM EDTA; 0.2% N-lauroylsarcosine; 1.5% (w/v) Ficoll 400 (Pharmacia Biotech, Uppsala, Sweden); 0.05% (w/v) bromophenol blue and 0.05% xylene cyanol] and were heated at 65°C for 3 minutes immediately prior to loading on the gel. The gels were stained with ethidium bromide (100 ng/ml) for 10 minutes and the DNA was visualised under UV light (Sambrook *et al.,* 1989).

III.2.1.7 *Preparation of virus stocks.*

Seed stocks of VV-HA-eotaxin were prepared by 2 rounds of amplification in 143B cells in 25 cm^2 and 175 cm^2 tissue culture flasks under MTAGG (TK^+) selection. 143B cells were infected with logarithmic dilutions of trypsinised virus (see section III.2.1.3) and were grown in F15 Complete for 2 days at 37°C/5% CO_2. After draining the media, cells were stained with 0.1% crystal violet/20% EtOH and the viral plaques counted.

Roller stocks of VV-HA-eotaxin and VV-HA-TK (Andrew *et al.*, 1986) were prepared in confluent monolayers of CV-1 cells that were grown in 850 cm^2 roller bottles. CV-1 cell were infected with 2 x 10^6 plaque forming units (pfu) of virus (seed stock) and were grown for 3 days on a roller at 37°C. The cells were then harvested using a cell scraper, pelleted by centrifugation (1800 x g for 5 minutes at 4°C) and resuspended in 15 mls of GSH. Roller stocks were stored at -70°C.

Roller stocks of virus were titred (see section III.2.1.8) using either F15 Complete medium, F15 Complete medium supplemented with MTAGG or F15 Complete medium supplemented with 25 μg/ml BrdU to determine total virus, TK^+ or TK^- viral titres, respectively.

III.2.1.8 *Viral titring using plaque assay.*

Viral suspensions were sonicated [2 x 10 seconds in ice water at 50 watts in a Sonifyer B12 cup sonicator (Branson Sonic Power Company, Danbury, CT., USA.)] and logarithmically diluted in GSH. Each dilution (100 μl) was added drop wise to drained 143B cells in a 6 well tissue culture plate that had been seeded at a density of 8 x 10^5 cells/well the previous day. After incubation at 37°C/5% CO_2 for 60 minutes, 2.5 mls of F15 Complete media was added to each well and then the plates were incubated for a further 2 days at 37°C/5% CO_2. The media was then removed and enough crystal violet (0.1% in 20% EtOH) was added to fully cover the well surface. Plates were air dried and viral plaques were counted. Viral titres were performed in duplicate and were determined from wells that contained 10-40 viral plaques.

III.2.2 Animals.

Male mice (C57BL/6, 6-8 wk of age) were used in all experiments.

III.2.3 The analysis of viral growth in murine lung.

VV-HA-TK and VV-HA-IL-5 were previously constructed and supplied by Dr Alistair Ramsay CJCSMR (Ramsay & Koharen G. 1993)

Ether-anaesthetised mice were given an intranasal (i.n.) inocula of 1 x 10^7 pfu of either VV-HA-eotaxin or control virus (VV-HA-TK) or 0.5 x10^7 pfu of both VV-HA-eotaxin and VV-HA-IL-5 in 20 µl of PBS. (Foster et al 1996). Mice were sacrificed on days 1 to 5 and 9 (VV-HA-eotaxin) or days 1 to 7 and 9 (VV-HA-IL-5 + VV-HA-eotaxin or VV-HA-TK) after viral inoculation and their lungs aseptically removed and stored at -70°C. Lungs were homogenised in 1 ml of PBS using a teflon homogeniser and sonicated as describe for viral suspension (section III.2.1.8). Viral titres were determined in monolayers of 143B cells in 6 well plates by logarithmic dilution (see section III.2.1.8).

III.2.4 The effect of VV-HA-eotaxin on the induction of pulmonary eosinophilia.

Mice were i.n. inoculated with 1 x 10^7 pfu of either VV-HA-eotaxin or VV-HA-TK. At peak viral growth (4 days post infection) the mice were sacrificed by cervical dislocation and the levels of leukocytes in the BALF determined (see section III.2.9) or histological examination of pulmonary tissues performed (see section III.2.12). Blood smears were taken daily post viral inoculations and the percentage of eosinophils in the total circulating leukocyte population determined by staining the slides with Giemsa-May-Grunwald. Routinely, 300-400 cells were counted per slide.

III.2.5 Effect of i.n. inoculation with VV-HA-eotaxin and/or VV-HA-IL-5 and i.v. injection of IL-5 on BALF eosinophilia.

Mice were given an i.n. inocula of 1 x 10^7 pfu of either VV-HA-eotaxin, VV-HA-IL-5 or VV-HA-TK or 0.5x 10^7 pfu each of VV-HA-eotaxin and VV-HA-IL-5 in combination. Four days later mice received an i.v. injection of either 200 pmol/kg of IL-5 or control vehicle (100 µl of 0.1%BSA/PBS). Blood samples were taken prior to and at 30 minutes post i.v. injection to confirm the induction of blood eosinophilia by IL-5. Blood eosinophil levels were determined using Discombe's method (section II.2.2). Twenty-four hours after i.v. injections the levels of eosinophils in the airways were assessed by BAL (section III.2.9).

III.2.6 Effect of i.n. inoculation with VV-HA-eotaxin and/or VV-HA-IL-5 and i.v. IL-5 on pulmonary function.

Mice were given an i.n. inocula of 0.5 x 10^7 pfu each of VV-HA-eotaxin and VV-HA-IL-5 or 1 x 10^7 pfu of VV-HA-TK (control virus). Four days later mice received an i.v. injection of 200 pmol/kg IL-5 in 100 µl of 0.1%BSA/PBS (pH 7.4). Seventy-two hours

after i.v. injections the mice were analysed for leukocyte infiltration into the airways (see section III.2.9), airways hyperreactivity to β-methylcholine (see section III.2.11) and changes to tissue structure (see section III.2.12).

III.2.7 Determining the effect of i.n. co-inoculation with VV-HA-eotaxin and VV-HA-IL-5 on pulmonary dysfunction during antigen inhalation.

Mice were exposed to aerosolized Ova (10 mg/ml) in saline or saline alone, for 3 x 30 minutes periods with 30 minutes rest between exposures, every second day for 8 days. Three days after the first exposure to Ova the mice were i.n inoculated with a combination of 0.5 x 10^7 pfu of VV-HA-eotaxin and VV-HA-IL-5 or 1 x 10^7 pfu of VV-HA-TK alone. Four days after viral inoculation the mice received an i.v. injection of 200 pmol/kg of IL-5 in 100 µl of PBS/0.1% BSA (pH 7.4). The infiltration of leukocytes into the airways was analysed 5 and 7 days after viral inoculations by BAL (section III.2.9). The presence of extracellular MBP (as a marker of eosinophil activation) in BALF supernatants was also assessed (section III.2.13). A late-phase airways hyperreactivity was tested for 7 days after viral inoculation, 48 hours after the last exposure to Ova (section III.2.11). Ova-specific serum antibodies were determined 5 days after viral inoculations (see section III.2.10). Histological analysis of pulmonary tissues was performed 5 and 7 days after viral inoculations (section III.2.12).

III.2.8 Determining the role of CD4 in VV-HA-IL-5- and VV-HA-eotaxin-induced airways hyperreactivity following repeated exposure to Ova aerosol.

Mice were i.n. inoculated with VV-HA-IL-5 and VV-HA-eotaxin at 0.5 x 10^7 pfu/virus and exposed to OVA aerosol as previously described (see section III.2.7). Three days prior to viral inoculations and 3 days post viral inoculations the mice were i.p. injected with 1 mg of anti-CD4 mAb (GK1.5) or 1 mg of rat isotype control IgG mAb (GL113). Airways hyperreactivity (see section III.2.11) and the leukocyte levels in the BALF (see section III.2.9) were determined 7 days after viral inoculation. The levels of Ova-specific serum IgG1 and total Ova-specific serum IgG were also determined (see section III.2.10) 7 days after viral inoculations.

III.2.9 The analysis and quantitation of leukocyte populations in the airway lumen by BAL.

Mice were killed by cervical dislocation and the trachea cannulated with a blunt 18 gauge needle. The lungs were then gently lavaged with 2 x 1 ml of HBSS and the lavage fluid placed on ice. The number of cells/ml of lavage fluid were determined using a

haemocytometer and 2 x 10^5 cells were cytocentrifuged and stained with Giemsa-May-Grunwald for differential cell counting. Leukocyte populations were identified by morphological criteria. Routinely, 300-400 cells were counted per slide.

III.2.10 The analysis Ova-specific antibody titres in sera and BALF by ELISA.

Flat bottom 96-well microtitre plates (Dynatech, Chantill, VA., USA.) were coated with either 100 μg/well Ova or 5 μg/well of either goat anti-mouse IgG_1, total IgG, IgA or IgE (Biosource International, Camarillo, CA., USA) in $NaHCO_3$ buffer (pH 9.6) and incubated at 4°C overnight. After washing 5 times with PBS/0.04% Tween-20 (SIGMA Chemical Co., St Louis, MO., USA.) the non-specific binding sites were blocked with 3% skim milk powder/PBS/0.04% Tween-20 for 1 hour at 37°C. Plates were then washed in PBS/0.04% Tween-20 and incubated with serial dilutions of either BALF or serum samples or standard murine IgG_1, total IgG, IgA or IgE (SIGMA Chemical Co., St Louis, MO., USA.). Following incubation at room temperature for 2 hours the plates were washed with PBS/0.04% Tween-20 and incubated with biotinylated goat anti-mouse IgG_1, total IgG, IgA or IgE (Biosource International, Camarillo, CA., USA.) in PBS/0.04% Tween-20 at room temperature for 1 hour. The plates were then washed with PBS and incubated with streptavidin-conjugated alkaline phosphatase (Amersham International plc., Buchinghamshire, UK.) for 1 hour at room temperature. Alkaline-phosphatase substrate solution (SIGMA Chemical Co., St Louis, MO., USA.) was then added and left to develop for 30 minutes at room temperature. Colour reactions were stopped by the addition of 50 μl of 0.1 M citric acid and the plates were read at 405 nm using a Microplate reader (BIO-TECH Instruments Inc., Winooski, VT., USA.).

III.2.11 Analysis of airways hyperreactivity to β-methylcholine.

Mice were anaesthetised by i.p. injection of 300 μl of a cocktail containing 60 mg/kg ketamine [Ketamil (Troy Laboratories Pty. Ltd., Smithfield, NSW., Australia)]/8 mg/kg xylazine [Rompun (Bayer Australia Ltd., Pymble, NSW., Australia)/7.2 mg/kg Acepromazine [Promex-2 (Apex Laboratories Pty. Ltd., St Mary's, NSW., Australia)] and the left or right lateral tail veil cannulated with a 25 gauge x 3/4 inch Winged Infusion set (Terumo Co-operation, Tokyo, Japan). The trachea was then exposed and cannulated with a blunt 18 gauge needle that was co-connected to a rodent ventilator (60 strokes/minute with a stroke volume of 1 ml) and a bronchospasm transducer (Ugo Basil-7020, Varese, Italy). The bronchospasm transducer was linked (Foster *et al*, 1996) to a Lab Mac/8 analysis station (AdInstruments, Sydney, Australia). The mice were maintained at 30°C and injected via the tail vein cannula with 50 μl of suxamethonium chloride [0.5 mg/kg (Astral Pharmaceuticals, Sydney, Australia)] to inhibit spontaneous

respiration. After a stable baseline was achieved, airway constriction was measured by monitoring changes in respiratory overflow volume during cumulative i.v. administration of β-methylcholine (12.5-1000 μg/kg) (SIGMA Chemical Co., St Louis, MO., USA.). Following the final dose of β-methylcholine, the tracheal cannula was totally occluded to determine the maximal occlusion.

III.2.12 Histological analysis of pulmonary tissues.

Transverse sections (approximately 4 mm wide) of lung were cut from the left lobe and fixed in 10% neutral buffered formalin for a minium of 24 hours. Samples were processed by the Histological Unit (John Curtin School of Medical Research ANU, Canberra, A.C.T., Australia). Briefly, fixed tissue was embedded in paraffin and 8 μm sections were cut onto glass slides. After heat affixing to the glass slide, the sections were deparaffinized in xylene, rehydrated using a gradient alcohol solutions and stained with Mayer's haematoxylin and eosin. Slides were mounted in DePeX mounting medium (BDH Laboratory Supplies, Poole, England) and coversliped. The sections were examined using light microscopy and were photographed using Ektachrome 64T tungsten colour film (Eastman Kodak Company, Rochester, NY., USA.).

III.2.13 The detection of free MBP in BALF using Western dotblot.

Polyvinylidene difluoride (PVDF) membranes were submerged in 100% methanol for 1 minute and then in TBS [20 mM Tris-HCl/150 mM NaCl (pH 8.0)] for 2 minutes. Membranes were placed on TBS soaked filter paper and 3 μl of each cell-free BALF sample was placed on the membrane. After drying at room temperature membranes were immersed in methanol and then TBS as described above. Membranes were then incubated with 1/2000 dilution of a polyclonal rabbit anti-mouse MBP (a gift from Dr G. J. Gleich, Mayo Clinic, Rochester, MI., USA.) in TBS/0.04% Tween-20 [polyoxyethylene-sorbitan monolaurate] (SIGMA Chemical Co., St Louis, MO., USA.) for 1 hour at room temperature on a rocker. After washing with TBS for 3 x 5 minutes on a rocker at room temperature, 0.2 μg/ml of goat anti-rabbit IgG alkaline-phosphatase conjugate was added and the membranes incubated for 1 hour at room temperature on a rocker. After washing the membranes with TBS for 3 x 5 minutes, Western Blue stabilised substrate for alkaline-phosphatase (Promega Corporation, Madison, WI., USA.) was added to cover the membrane and the reaction developed for 3 minutes at room temperature. Colour reactions were arrested by washing the membrane in PBS.

III.3 RESULTS.

III.3.1 The sustained expression of eotaxin induces pulmonary eosinophilia in mice.

The kinetics of VV-HA-eotaxin growth and clearance in the lungs of mice that were inoculated with 1 x 10^7 pfu were similar to that observed for the control virus (VV-HA-TK) (figure III.2). The peak viral titres for both VV-HA-eotaxin and VV-HA-TK were observed 4 to 5 days after inoculation at the level of 10^6 pfu/lung. Viral titres subsequently declined to 10^3 pfu/lung by day 7. Neither virus was detected (level of detection < 10^2 pfu/lung) in the lungs 9 days after viral inoculation (figure III.2). Four days post viral inoculation (at peak viral burden), the mice that received VV-HA-eotaxin displayed a significant BALF eosinophilia (4.01 x $10^3 \pm 0.62$ x 10^3 eosinophils/ml BALF), in contrast to mice inoculated with control virus (VV-HA-TK) (0.2 x 10^3 eosinophils/ml BALF). The level of eosinophils in the BALF of VV-HA-TK inoculated mice were the same as those of non-treated mice (figures III.3).

III.3.2 VV-HA-eotaxin and VV-HA-IL-5 co-inoculation induces a pronounced pulmonary eosinophilia in mice that is further amplified in the presence of i.v. IL-5.

Viral growth and viral clearance in the lungs of mice was similar for groups that received VV-HA-eotaxin and VV-HA-IL-5 each at half dose (0.5 x 10^7 pfu) to those that received a full dose (1 x 10^7 pfu) of the control virus (VV-HA-TK) (figure III.4). Viral growth and clearance of VV-HA-IL-5 in the lungs of mice that were given an i.n. inocula of 1 x 10^7 pfu has previously been reported (Ramsay and Kohonen-Corish, 1993) and was not significantly different from that of the control virus (VV-HA-TK). Thus, neither eotaxin alone (figure III.2) or in combination with IL-5, significantly effects vaccinia viral growth or viral clearance mechanisms in the lungs of mice.

VV-HA-eotaxin (0.5 x 10^7 pfu) and VV-HA-IL-5 (0.5 x 10^7 pfu) together induced a significantly greater BALF eosinophilia when compared with groups that received either of the viruses alone at the same total viral load (1 x 10^7 pfu) (figure III.5). VV-HA-IL-5 induced a BALF eosinophilia equivalent to VV-HA-eotaxin (figure III.5).

In Chapter II it was shown that the i.v. injection of IL-5 enhanced the cutaneous accumulation of eosinophils in mice in response to eotaxin or IL-5 (see section II.3.2). Similarly, i.v. administration of IL-5 also amplified eosinophil recruitment to pulmonary tissues in response to VV-HA-IL-5 and/or VV-HA-eotaxin infection (figure III.5).

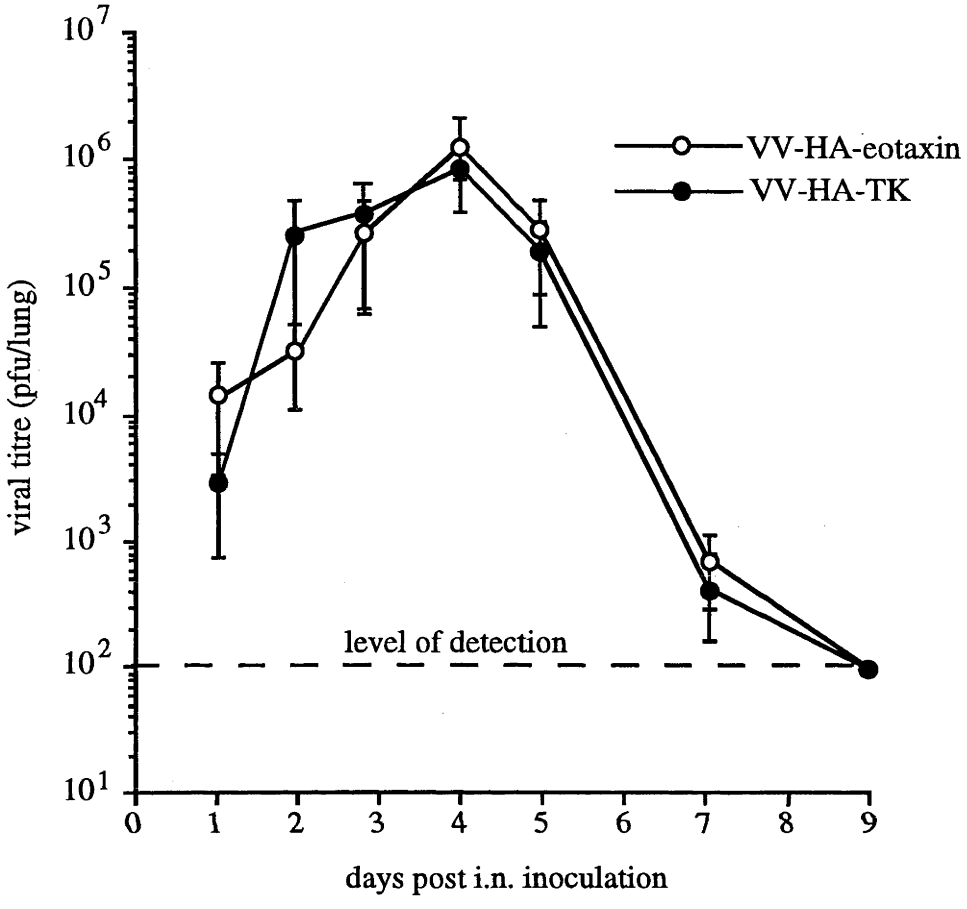


Figure III.2 ***Kinetics of viral growth and clearance in the lungs for VV-HA-eotaxin in comparison to VV-HA-TK.***

Mice were inoculated with 1 x 10^7 pfu of VV-HA-eotaxin or VV-HA-TK. Data represents mean viral titre/lung ± SEM of groups of 4 mice in duplicate as determined by viral plaque assay. The kinetics of VV-HA-eotaxin growth and clearance in the lungs of mice were the same as that for the VV-HA-TK.

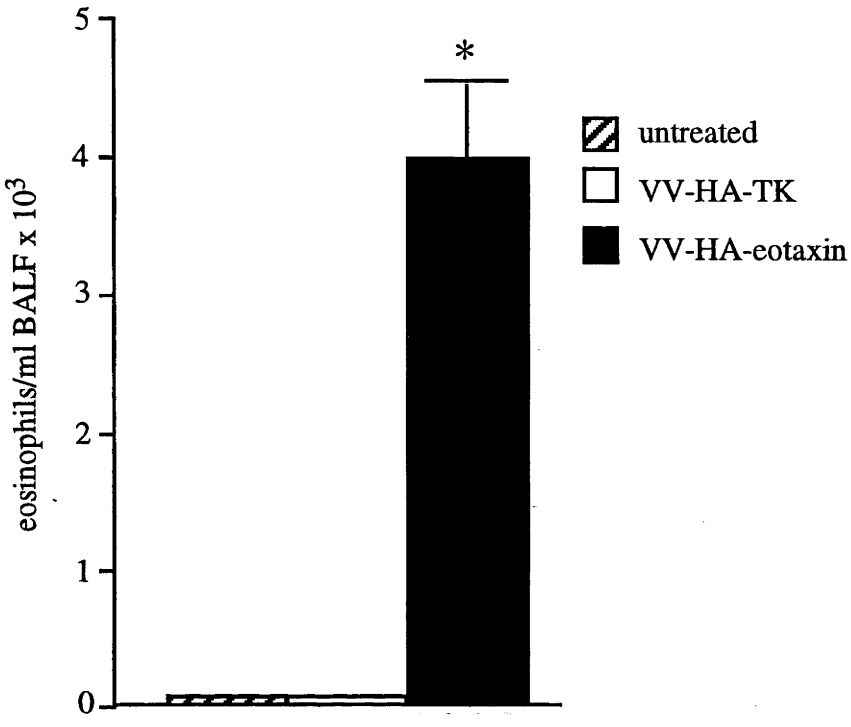


Figure III.3 ***VV-HA-eotaxin induces BALF eosinophilia.***

Mice were inoculated with 1 x 10^7 pfu of either VV-HA-eotaxin or VV-HA-TK (control virus) and the levels of eosinophils in the BALF analysed 4 days later. Mice inoculated with VV-HA-eotaxin had significantly higher levels of eosinophils in the BALF compared with VV-HA-TK (control virus) inoculated mice. Data represents mean eosinophils/ml BALF ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. $^*P < 0.001$ when compared with VV-HA-TK.

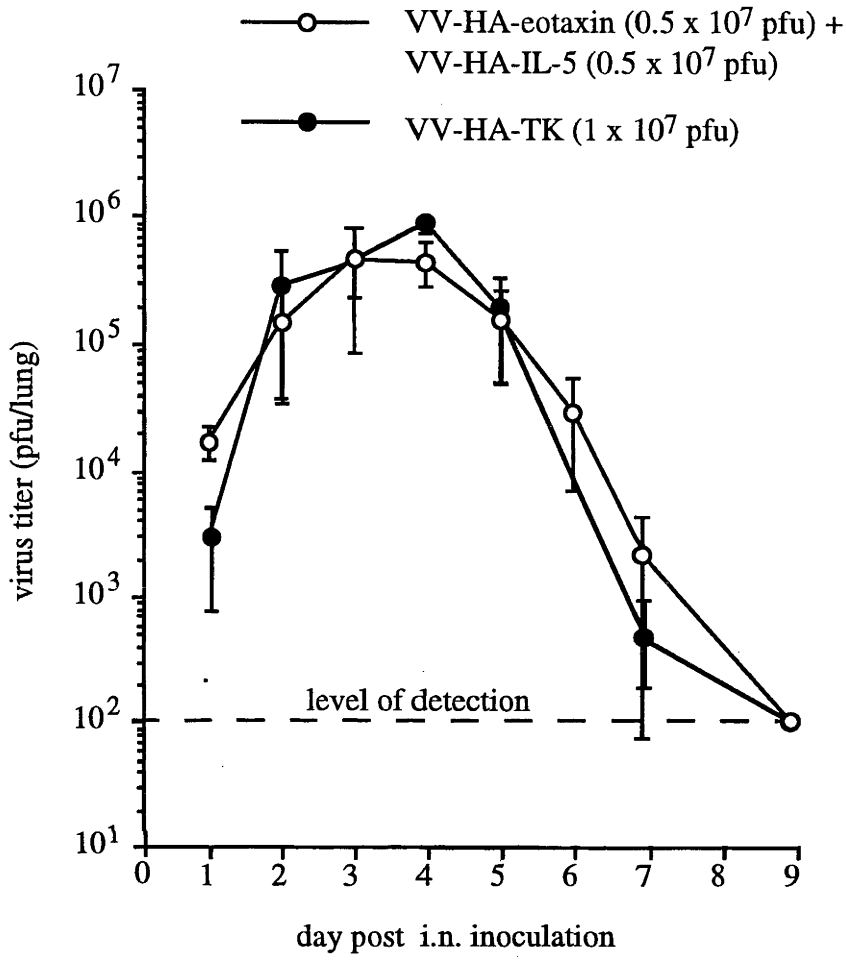


Figure III.4 ***Kinetics of VV-HA-eotaxin and VV-HA-IL-5 growth and clearance in the lungs.***

Viral growth and clearance in the lungs of mice that received an i.n. co-inocula of VV-HA-eotaxin (0.5 x 10^7 pfu) and VV-HA-IL-5 (0.5 x 10^7 pfu) were simiar to that for the VV-HA-TK (control virus) at the same total viral load (1 x 10^7 pfu/lung). Data represents mean viral titre/lung ± SEM of groups of 4 mice in duplicate as determined by viral plaque assay.

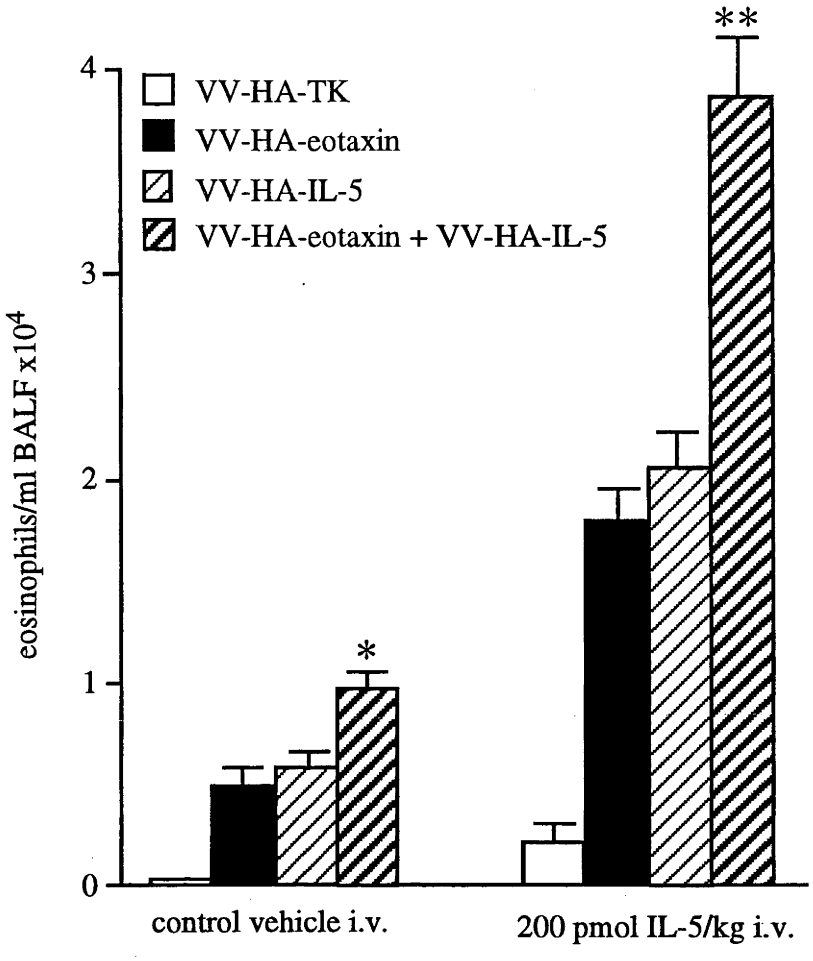


Figure III.5 ***VV-HA-IL-5 and VV-HA-eotaxin together are potent inducers of BALF eosinophilia in mice.***

Mice were given an i.n. co-inocula of both VV-HA-eotaxin (0.5 x 10^7 pfu) and VV-HA-IL-5 (0.5 x 10^7 pfu), or a single inocula (1 x 10^7 pfu) of of VV-HA-eotaxin, VV-HA-IL-5, or VV-HA-TK (control virus). Four days later the mice received an i.v. injection of 200 pmol/kg IL-5 or control vehicle [100 µl of 10 mM PBS/0.1% BSA (pH 7.4)]. Mice were sacrificed 24 hours after i.v. injections and their airways lavaged (BAL) with 2 x 1 ml of HBSS. Data represents mean eosinophils/ml BALF ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. $*P < 0.05$ and $**P < 0.01$ when compared with VV-HA-eotaxin or VV-HA-IL-5 alone with i.v. control vehicle.

The BALF eosinophilia induced by VV-HA-eotaxin and/or VV-HA-IL-5 was further enhanced 5 days after inoculation when IL-5 (200 pmol/kg i.v.) was administered 24 hours earlier (figure III.5). Interestingly, i.v. IL-5 also significantly increased the levels of eosinophils in the BALF in response to VV-HA-TK (control virus). Although the levels of each of the viruses in the lungs were similar 5 days after viral inoculations, VV-HA-eotaxin (0.5×10^7 pfu) and VV-HA-IL-5 (0.5×10^7 pfu) induced a BALF eosinophilia that was significantly higher than that induced by either virus in isolation (at the same total viral load) (figure III.5).

The histological examination of lung sections from all groups of vaccinia virus inoculated mice revealed a considerable cellular infiltrate in the perivascular and the peribronchiolar regions (figure III.6). In contrast to VV-HA-TK (control virus), VV-HA-eotaxin and VV-HA-IL-5 induced tissue eosinophilia in perivascular (not shown) and peribronchiolar regions (figure III.7). Notably, eosinophils in the peribronchiolar regions were specifically localised to areas directly under the smooth muscle layer. In contrast, few eosinophils were found in the region between the airways smooth muscle and the bronchial epithelium.

Although VV-HA-IL-5 and VV-HA-eotaxin selectively induced a significant BALF eosinophilia ($P > 0.001$ compared with VV-HA-TK) (figure III.8a) they did not induce airways hyperreactivity (figure III.8b). Furthermore, no MBP was detected in the BALF following VV-HA-IL-5 and VV-HA-eotaxin or VV-HA-TK inoculation (table III.1). None of the viruses causes significant damage (epithelial shedding) to the airways (figure III.7).

III.3.3 VV-HA-eotaxin and VV-HA-IL-5, but not VV-HA-TK, induces airways hyperreactivity to β–methylcholine in mice following repeated exposure to aeroallergen.

Ova aerosol exposure did not affect the levels of eosinophils, lymphocytes or neutrophils in the BALF, either in the presence of VV-HA-IL-5 and VV-HA-eotaxin or VV-HA-TK (figure III.9a). However, macrophage levels in the BALF were significantly higher ($P < 0.05$) following Ova aerosol exposure when compared with saline aerosol exposed controls (figure III.9a).

Following multiple exposures to Ova aerosol, VV-HA-eotaxin and VV-HA-IL-5 induced airways hyperreactivity to i.v. β-methylcholine (figure III.9b). In contrast, these viruses had no effect of airways reactivity following multiple exposures to saline aerosol (figure III.9b). VV-HA-TK (control virus) failed to induce airways hyperreactivity following multiple exposures to either Ova or saline aerosol (figure III.9b).

MBP was only detected in the BALF of the VV-HA-IL-5 and VV-HA-eotaxin co-inoculated group following repeated exposure to Ova aerosol. MBP was detected 5 days after viral inoculation (table III.1) and airways hyperreactivity was detected two days later (figure III.9b). Interestingly, no MBP was detectable in the BALF when airways hyperreactivity developed.

Although the repeated exposure of mice to aerosolized Ova did not induce pulmonary eosinophilia to the same magnitude as that observed in mice during severe allergic airways inflammation (Foster *et al.,* 1996), it did elicit a weak immune response 5 days after viral inoculation, as detected by the presence of Ova-specific serum IgG. The antibodies were predominantly of the IgG_1 isotype (figure III.10) and no Ova-specific BALF IgA or serum IgE were detectable in these mice.

Although VV-HA-IL-5 and VV-HA-eotaxin induced airways hyperreactivity in the presence of repeated exposures to Ova aerosol, no visible damage to the structure of the airways was observed at 5 and 7 days after viral inoculation (not shown). Similar to the BALF, eosinophil levels in the lungs after VV-HA-IL-5 and VV-HA-eotaxin inoculation were not different between Ova and saline aerosol treatments (figure III.9a). Furthermore, the eosinophils that were present in the lungs of all of these groups were consistently and predominantly localised to regions under the airways smooth muscle as previously observed (figure III.7).

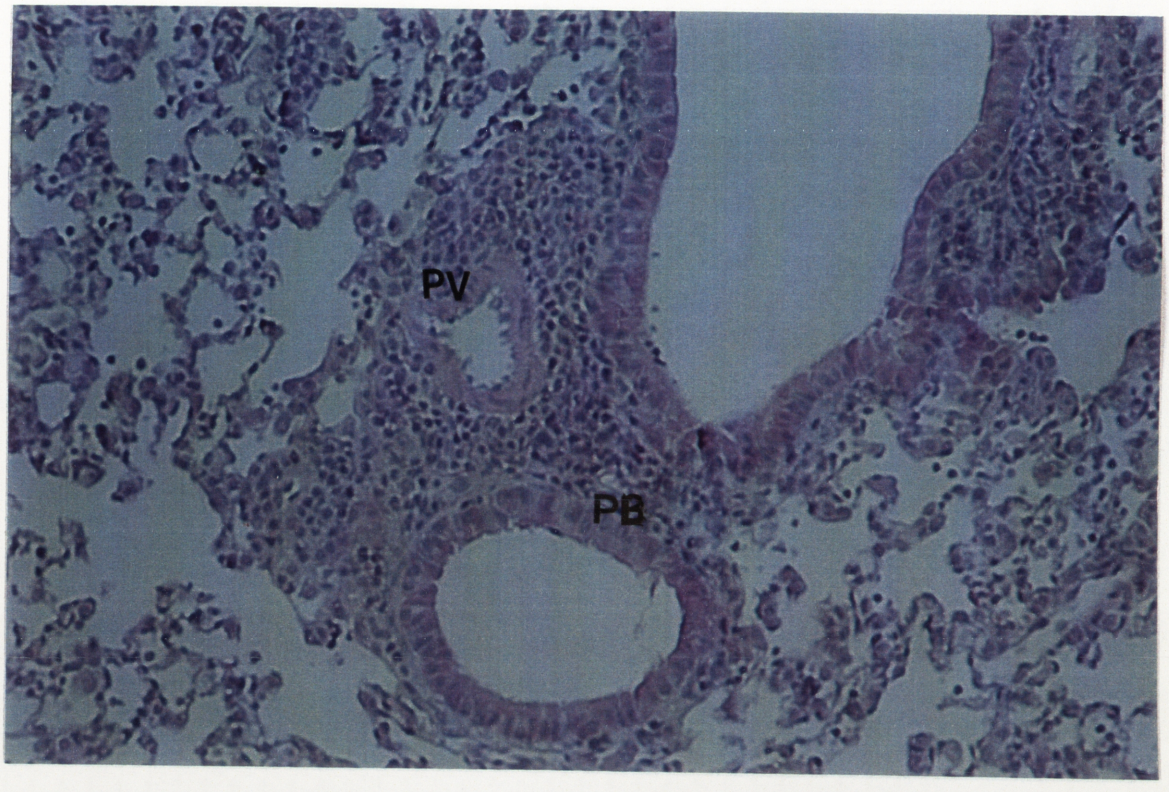
III.3.4 The development of airways hyperreactivity in VV-HA-IL-5 and VV-HA-eotaxin inoculated mice following exposure to Ova aerosol is CD4 dependent.

To test the role of CD4 in the induction of airways hyperreactivity by VV-HA-IL-5 and VV-HA-eotaxin following repeated exposure to Ova aerosol, mice were treated i.p. with anti-CD4 mAb. Anti-CD4 mAb treatments abolished the production of Ova specific serum IgG1 and total Ova-specific serum IgG (figure III.11a) and the development of airways hyperreactivity (figure III.11b). Furthermore this treatment did not affect individual leukocyte levels in the BALF (figure III.12).

Figure III.6 ***Peribronchial and perivascular infiltration of leukocytes in vaccinia virus infected lungs.***

(a) There was a substantial infiltration of leukocytes in the perivascular (PV) and peribronchial (PB) regions of lungs 5 days after viral (VV-HA-TK) inoculation when compared to (b) the lungs from untreated controls. Photographs (100 x magnification) are of 8 μM paraffin sections stained with Giemsa-May-Grunwald.

(a)



(b)

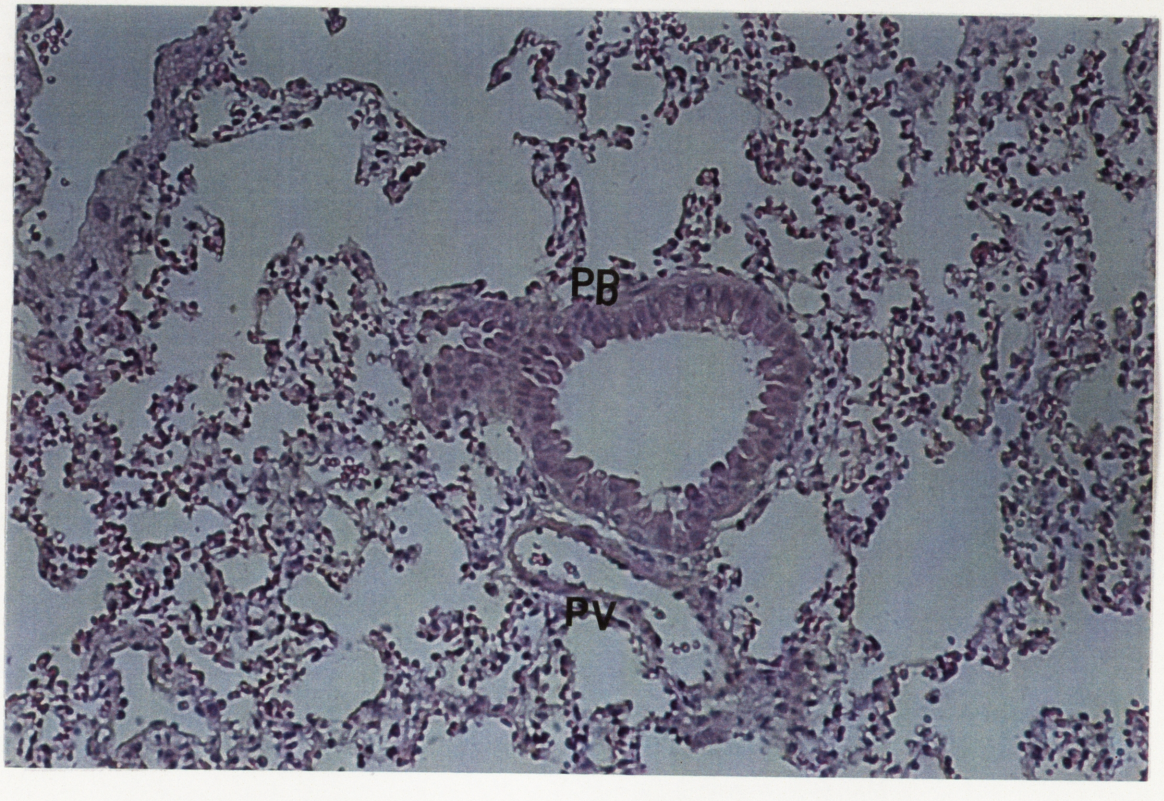


Table III.1 ***MBP levels in the BALF of vaccinia virus inoculated mice following multiple exposures to Ova or saline aerosol.***

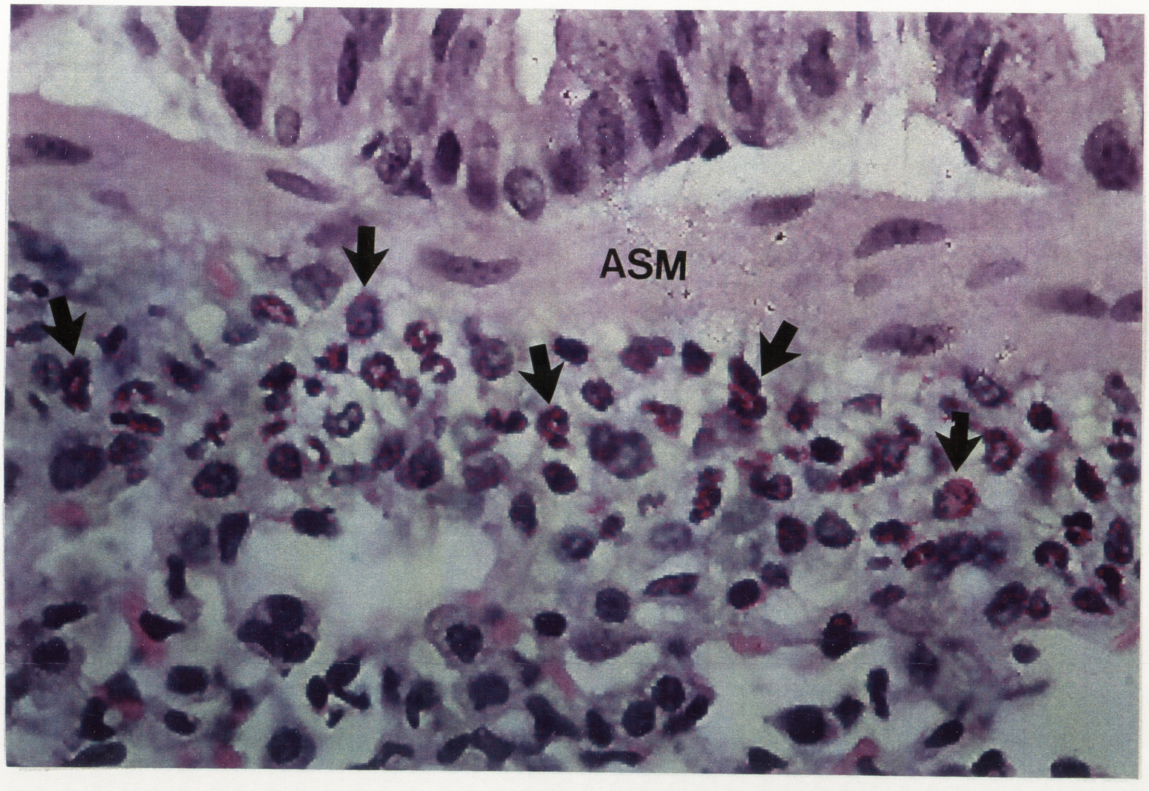
	Saline aerosol		Ova aerosol	
Viral inocula	Day 5	Day 7	Day 5	Day 7
VV-HA-TK(1.0 x 10^7 pfu)	X	X	X	X
VV-HA-IL-5 (0.5 x 10^7 pfu)+ VV-HA-eotaxin (0.5 x 10^7 pfu)	X	X	√	X

The presence of free MBP in the BALF of mice inoculated with VV-HA-IL-5 (0.5 x 10^7 pfu) and VV-HA-eotaxin (0.5 x 10^7 pfu) or VV-HA-TK (1.0 x 10^7 pfu) was analysed in sample taken from mice 5 and 7 days after viral inoculation and was determined by western dot blot. Ticks represent positive MBP staining on immuno dot blots and a cross denotes that MBP was not detected. MBP was only detected in the BALF of Ova aerosol exposed VV-HA-IL-5 and VV-HA-eotaxin inoculated mice 5 days after virus inoculation.

Figure III.7 ***VV-HA-eotaxin and VV-HA-IL-5 induce pulmonary eosinophilia.***

Mice were co-inoculated with VV-HA-IL-5 (0.5 x 10^7 pfu) and VV-HA-eotaxin (0.5 x 10^7 pfu) or VV-HA-TK (1 x 10^7 pfu). Five days later the mice were sacrificed and the lungs examined. Photographs (400 x magnification) are of 8 μM paraffin sections stained with Giemsa-May-Grunwald. (a) Eosinophils were present in peribronchial regions following VV-HA-IL-5 and VV-HA-eotaxin inoculation and were specifically localised to areas adjacent to the airways smooth muscle layer (ASM). (b) In contrast, few eosinophils were found in this region following VV-HA-TK (control virus) inoculation.

(a)



(b)

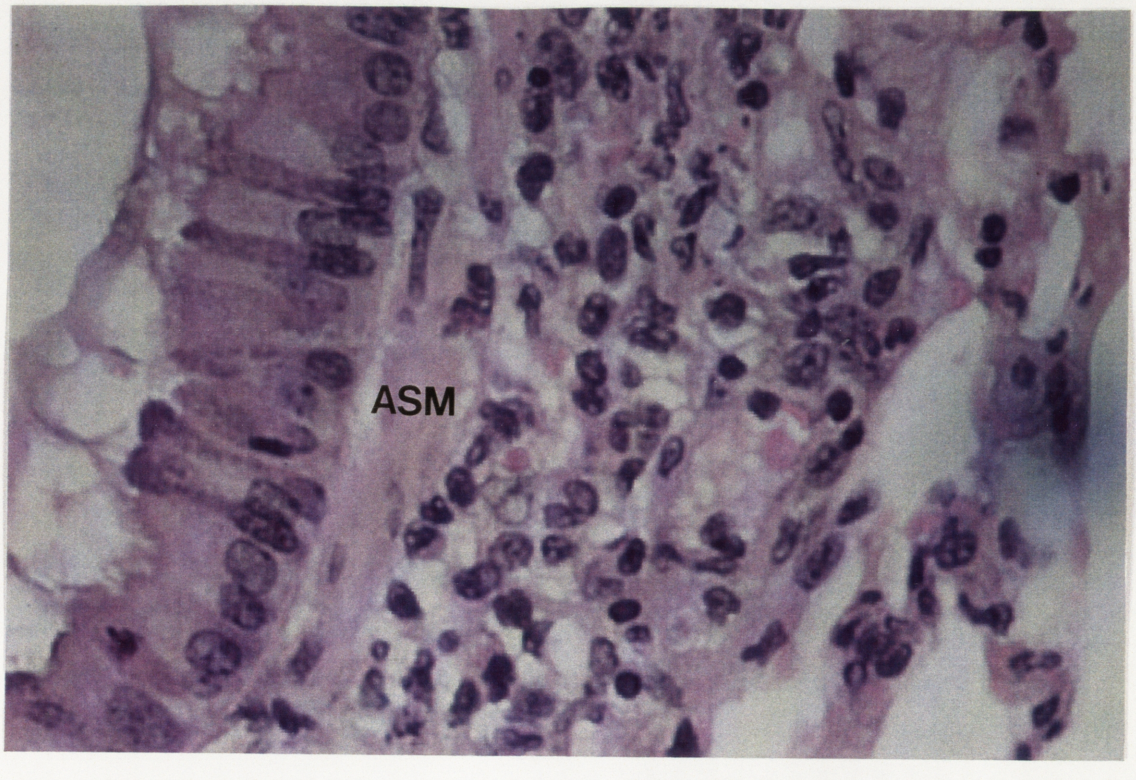


Figure III.8 ***Co-inoculation with VV-HA-eotaxin and VV-HA-IL-5 induces a selective recruitment of eosinophils into the lungs but does not induce airways hyperreactivity.***

Mice were inoculated with VV-HA-eotaxin (0.5×10^7 pfu) and VV-HA-IL-5 (0.5×10^7 pfu) or VV-HA-TK (1×10^7 pfu) and four days later were given an i.v. injection of 200 pmol/kg IL-5. Airway hyperreactivity and leukocyte levels in the BALF were analysed 7 days after viral inoculation. (a) Leukocyte levels in BALF. Data represents mean cells [eosinophils (eos), neutrophils (neuts), lymphocytes (lymphs) or macrophages (m/phages)] per ml of BALF ± SEM of groups of 5 mice. (b) Measurement of airways reactivity to β-methylcholine. Airways reactivity was measured by monitoring i.v. β-methylcholine-induced changes in respiratory overflow volume. Ova sensitised/challenged mice (Foster *et al.*, 1996) and non-treated mice were used as positive and negative controls, respectively. Data represents mean percentage airway occlusion ± SEM of groups of 6 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) $^*P < 0.001$ when compared with eosinophils levels in VV-HA-TK infected mice. (b) No significant difference in means between VV-HA-eotaxin/VV-HA-IL-5 and VV-HA-TK or between either of these groups and a non-treated (negative control) group at the same doses of β-methylcholine.

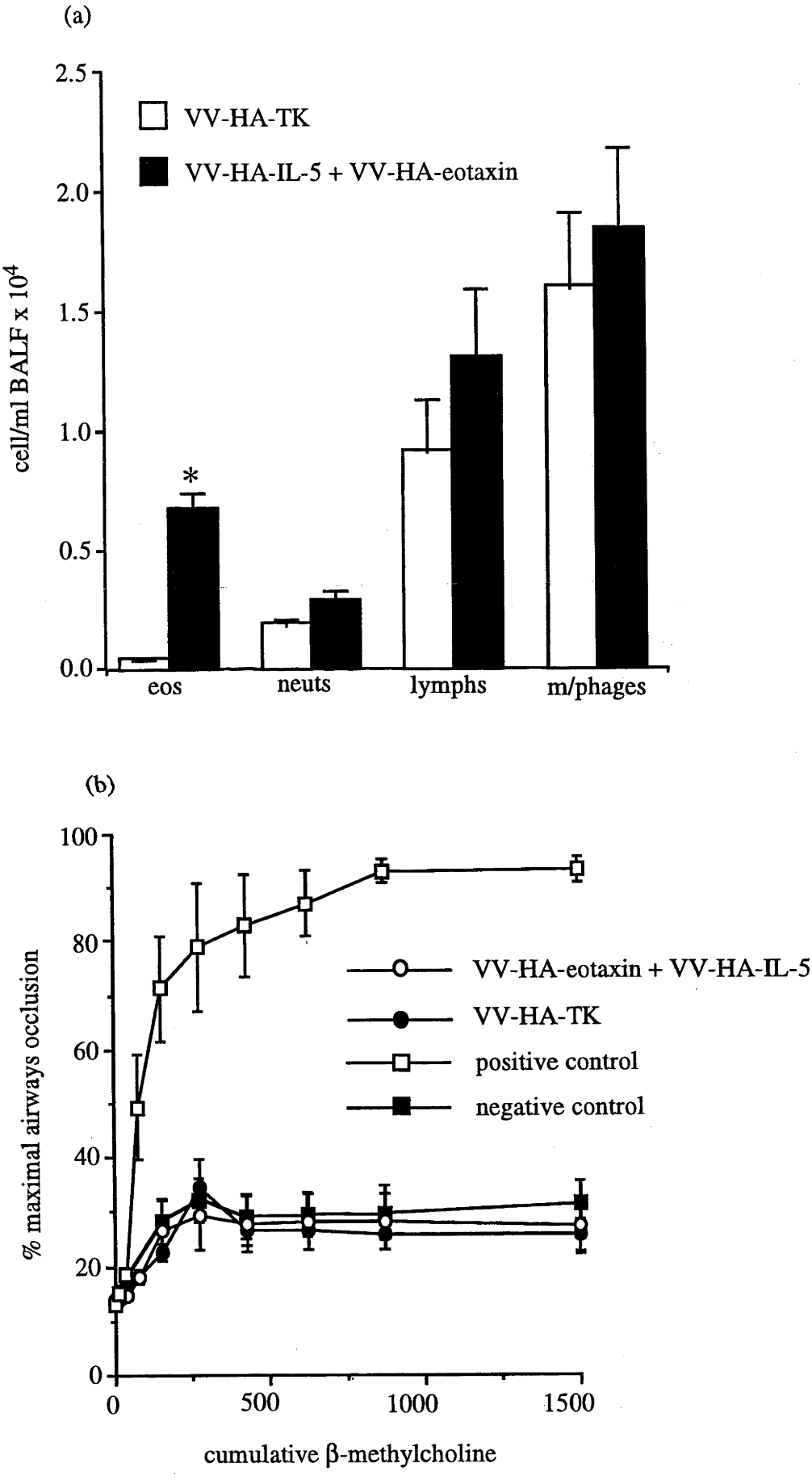
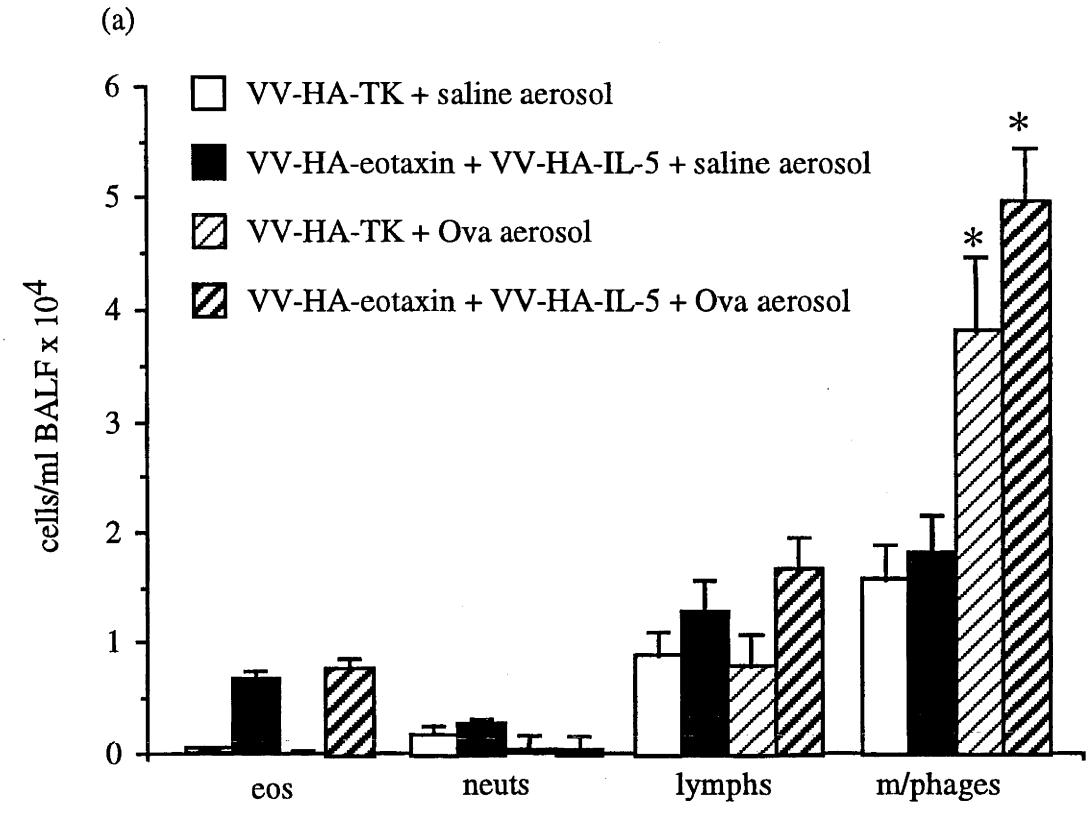
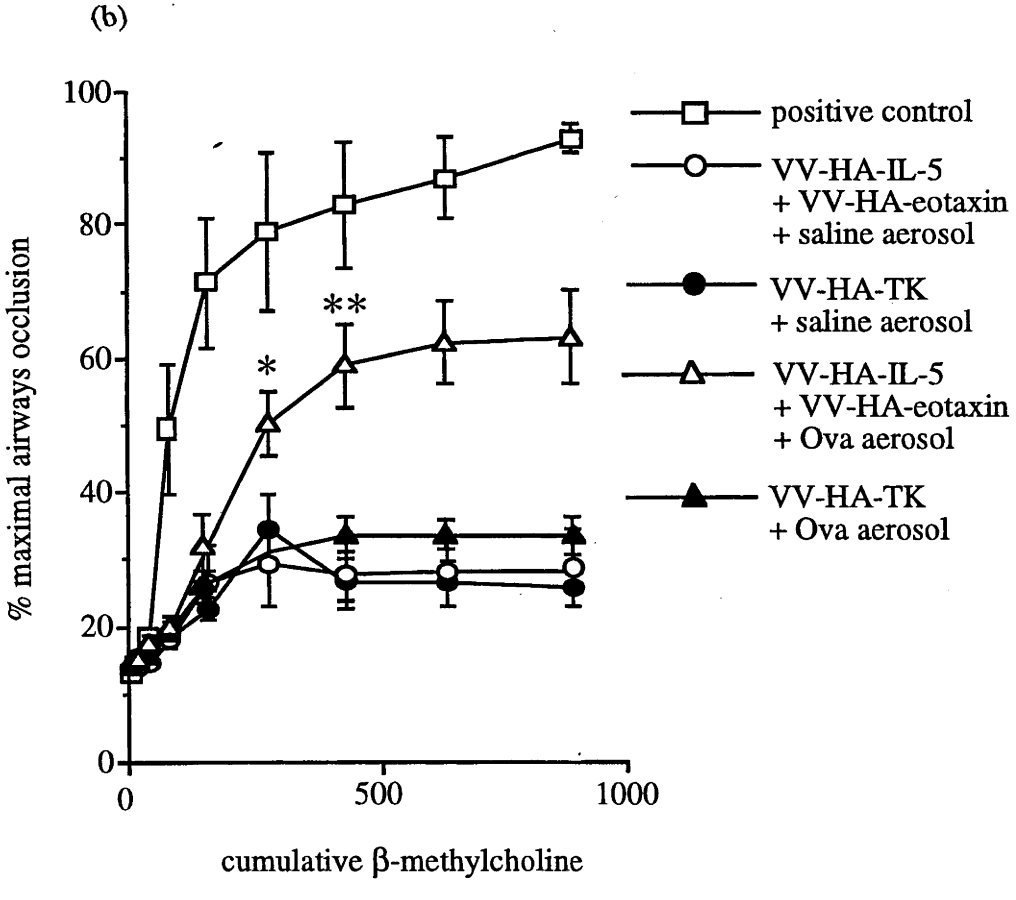

(a)
2.5
2.0
1.5
1.0
0.5
0.0
cell/ml BALF x 10^4
VV-HA-TK
VV-HA-IL-5 + VV-HA-eotaxin
*
eos
neuts
lymphs
m/phages
(b)
100
80
60
40
20
0
% maximal airways occlusion
VV-HA-eotaxin + VV-HA-IL-5
VV-HA-TK
positive control
negative control
0
500
1000
1500
cumulative β-methylcholine

Figure III.9 ***The repeated exposure of mice to Ova aerosol induces late-phase airways hyperreactivity in mice inoculated with VV-HA-eotaxin and VV-HA-IL-5 but not with VV-HA-TK, without changing BALF leukocyte populations.***

(a) Leukocyte levels in BALF 7 days after VV-HA-eotaxin and VV-HA-IL-5 or VV-HA-TK inoculation following multiple exposures to Ova or saline aerosol. Data represents mean eosinophils (eos), neutrophils (neuts), lymphocytes (lymphs) or macrophages (m/phages) per ml of BALF ± SEM of groups of 5 mice. (b) Airways reactivity was measured by monitoring i.v. β-methylcholine-induced changes in respiratory overflow volume. Ova sensitised/challenged mice (Foster *et al.*, 1996) were used as a positive control. Data represent the mean percentage airway occlusion ± SEM of groups of 6 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) $*P < 0.05$ when compared with groups that were exposed to saline aerosol. (b) $*P < 0.05$ and $** P < 0.01$ when compared with VV-HA-TK + Ova aerosol and VV-HA-IL-5 + VV-HA-eotaxin + saline aerosol groups.


(a)
VV-HA-TK + saline aerosol
VV-HA-eotaxin + VV-HA-IL-5 + saline aerosol
VV-HA-TK + Ova aerosol
VV-HA-eotaxin + VV-HA-IL-5 + Ova aerosol
cells/ml BALF x 10^4
0
1
2
3
4
5
6
*
*
eos
neuts
lymphs
m/phages


(b)
% maximal airways occlusion
0
20
40
60
80
100
*
**
0
500
1000
cumulative β-methylcholine
positive control
VV-HA-IL-5
+ VV-HA-eotaxin
+ saline aerosol
VV-HA-TK
+ saline aerosol
VV-HA-IL-5
+ VV-HA-eotaxin
+ Ova aerosol
VV-HA-TK
+ Ova aerosol

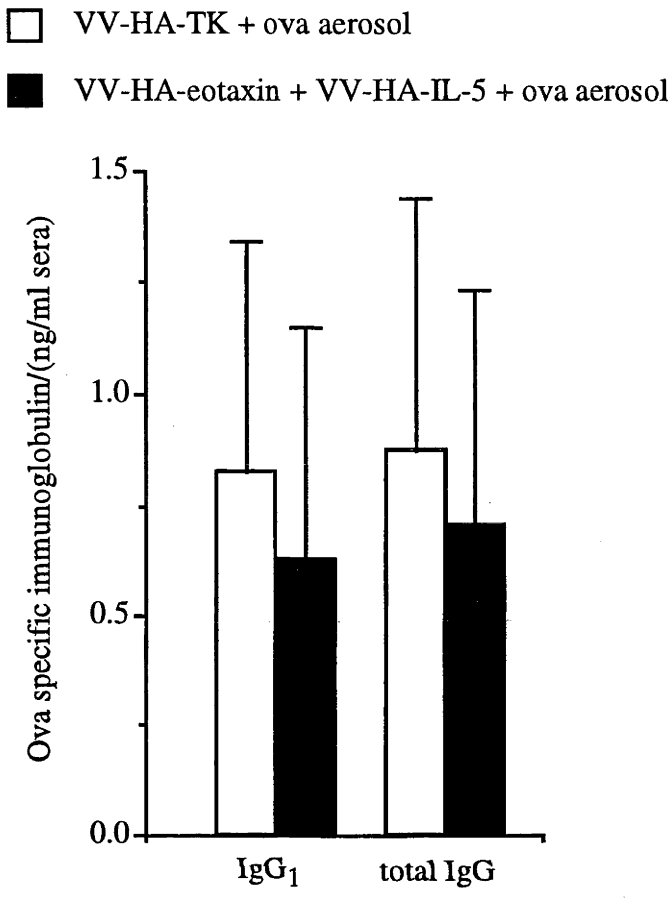
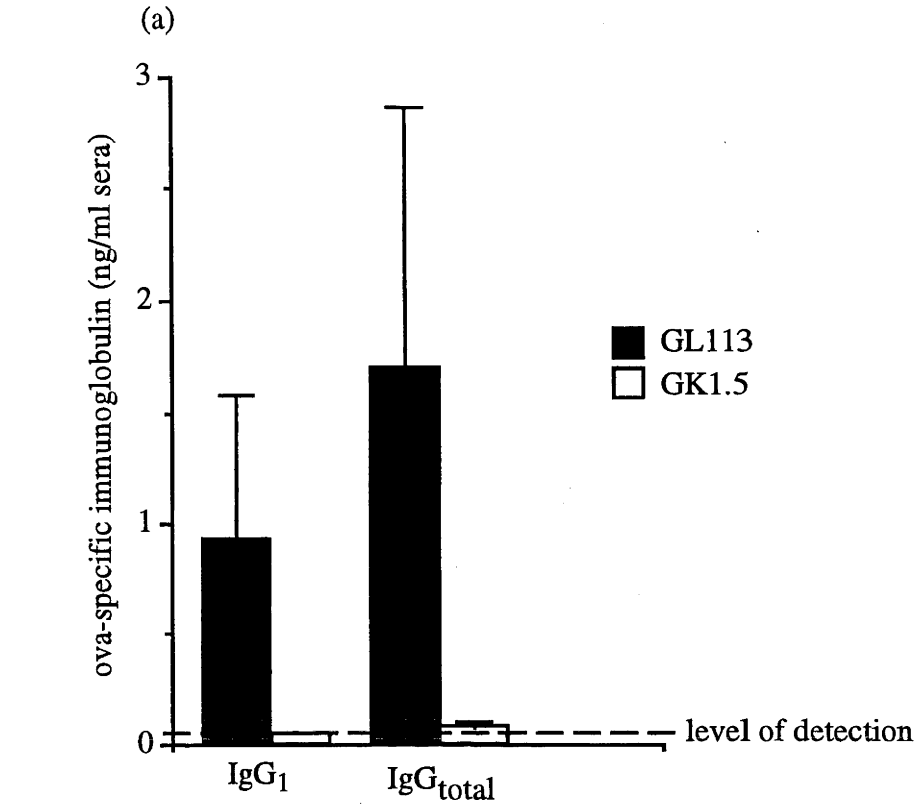


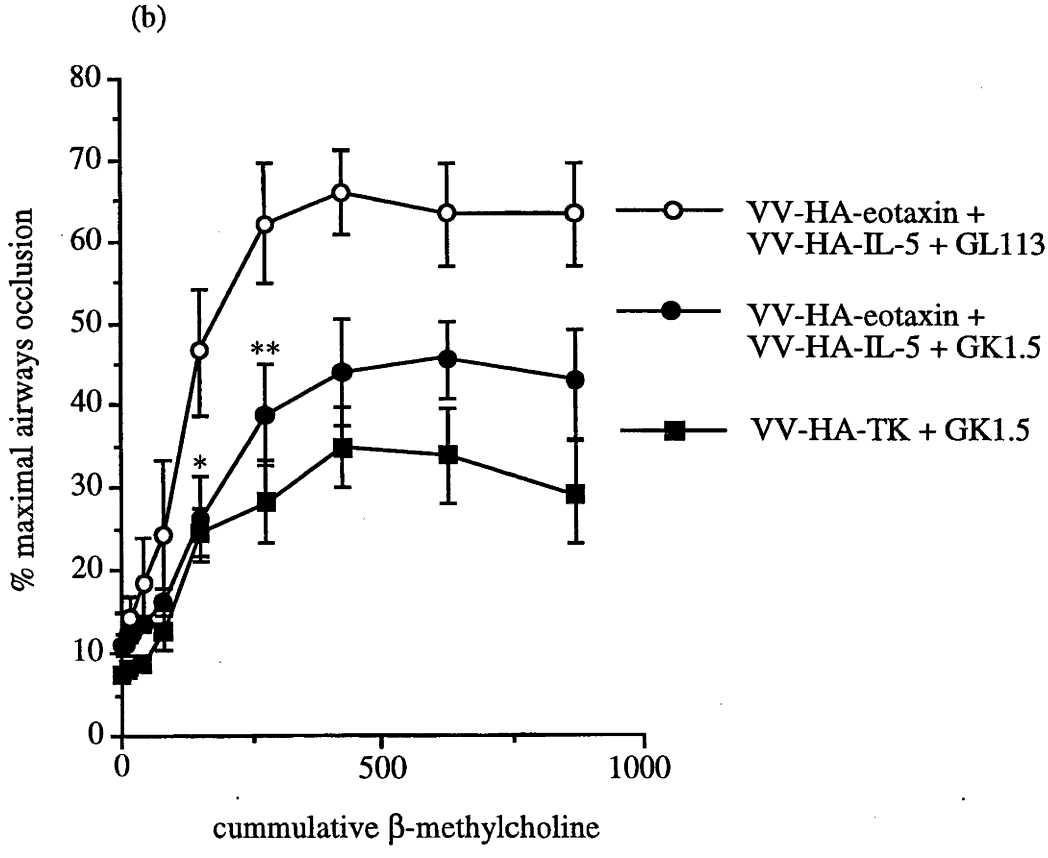
Figure III.10 ***The level of Ova-specific IgG_1 in comparison to total Ova-specific IgG in sera of mice exposed to Ova aerosol.***

Levels of Ova-specific serum IgG_1 and total Ova-specific serum IgG in VV-HA-IL-5 and VV-HA-eotaxin or VV-HA-TK inoculated mice following repeated exposure to Ova or saline aerosol. Serum samples were analysed 5 days after viral inoculation by ELISA. Data represents mean ng/ml sera of (a) Ova-specific IgG and (b) Ova-specific IgG_1 ± SEM of groups of 5 mice. Each sample was analysed in duplicate by serial dilution. No Ova-specific IgG was detectable in groups that were exposed to saline aerosol (not shown).

Figure III.11 ***Treatment of mice with anti-CD4 mAb abolishes the production of Ova-specific IgG1 and the development of late-phase airways hyperreactivity induced by VV-HA-eotaxin and VV-HA-IL-5 following repeated exposure to Ova aerosol.***

(a) Anti-CD4 mAb (GK1.5), but not control mAb (GL113) treatment abolished the production of Ova-specific IgG_1 and total Ova-specific IgG in the sera of VV-HA-eotaxin and VV-HA-IL-5 inoculated mice that were repeatedly exposed to Ova aerosol. Data represent mean ng/ml of sera of (a) total Ova-specific serum IgG and Ova-specific serum $IgG_1 \pm$ SEM of groups of 5 mice. Each sample was analysed by ELISA in duplicate by serial dilution. (b) Late-phase airways reactivity was measured by monitoring i.v. β-methylcholine-induced changes in respiratory overflow volume. Anti-CD4 mAb treatment abolished Ova aerosol-induced airways hyperreactivity in VV-HA-IL-5 and VV-HA-eotaxin inoculated mice. Data represent mean percentage airway occlusion ± SEM of groups of 6 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (b) $^{*}P < 0.05$ and $^{**}P < 0.02$ when compared with GL113 treated mice.


(a)
ova-specific immunoglobulin (ng/ml sera)
3
2
1
0
GL113
GK1.5
level of detection
IgG1
IgGtotal


(b)
% maximal airways occlusion
80
70
60
50
40
30
20
10
0
0
500
1000
cummulative β-methylcholine
*
**
VV-HA-eotaxin +
VV-HA-IL-5 + GL113
VV-HA-eotaxin +
VV-HA-IL-5 + GK1.5
VV-HA-TK + GK1.5

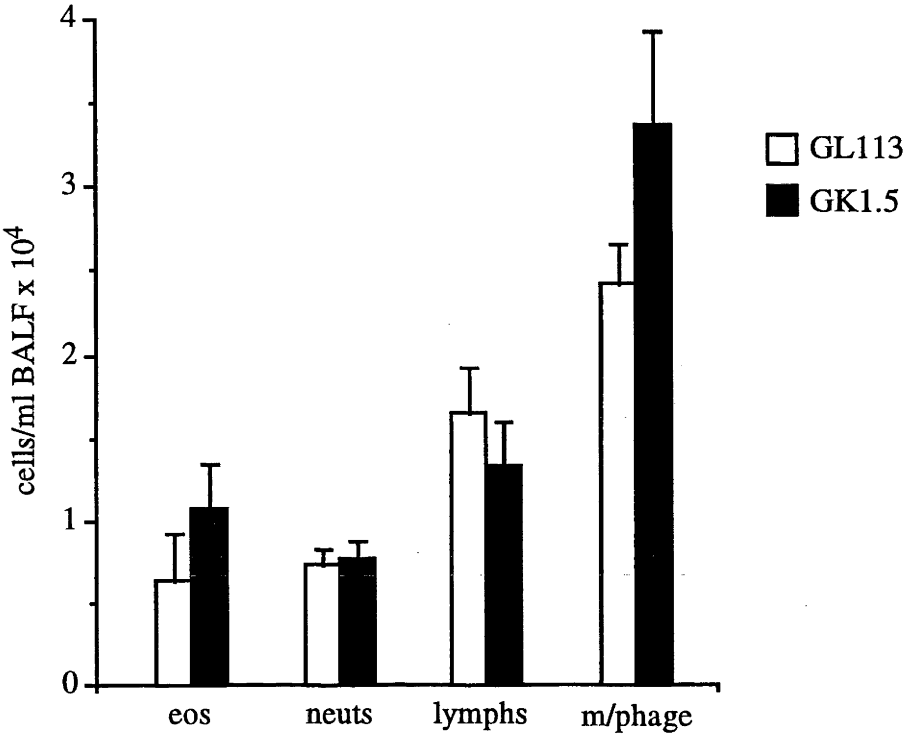


Figure III.12 ***Leukocyte levels in BALF 7 days after VV-HA-eotaxin and VV-HA-IL-5 inoculation in the presence of Ova aerosol and following treatment with anti-CD4 mAb.***

Mice were inoculated with VV-HA-IL-5 and VV-HA-eotaxin, repeatedly exposed to Ova aerosol and i.p. injected with anti-CD4 mAb (GK1.5) or isotype control mAb (GL113). Data represents mean eosinophils (eos), neutrophils (neuts), lymphocytes (lymphs) or macrophages (m/phages) per ml of BALF 7 days after viral inoculation ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. No significant differences were detected between the means of any leucocyte population following GK1.5 treatment when compared with groups treated with GL113.

III.4 DISCUSSION.

Although a single exposure of mice to recombinant eotaxin did not elicit a BALF eosinophilia (Rothenberg *et al.,* 1996), the sustained *in vivo* expression of this cytokine by recombinant vaccinia virus induced a significant BALF eosinophilia. IL-5, expressed by recombinant vaccinia virus also potently induced the accumulation of eosinophils in pulmonary tissues. Furthermore, the co-expression of these two cytokines significantly amplified pulmonary eosinophilia. The recent characterisation of mice where IL-5 was specifically expressed by the airway epithelium (under the control of the CC10 promoter), has indicated a role for this cytokine in the trafficking of eosinophils in the airways (Lee *et al.,* 1997). Both IL-5 and eotaxin have been shown to have important roles in eosinophil trafficking during allergic pulmonary inflammation in mice (Foster *et al.,* 1996; Rothenberg *et al.,* 1997). Results presented in this Chapter suggest that these two cytokines act co-operatively to regulate pulmonary eosinophilia and such interactions may also operate in the allergic lung. Furthermore, IL-5 and eotaxin appeared to operate synergistically at equivalent levels of virus.

Th_2 type responses to *N. brasiliensis* (Nb) infection are impaired in IL-4 deficient mice (Kopf *et al.,* 1993). Although elevated levels of eotaxin expression are still induced in the absence of IL-4 in these animals in response to Nb infection, both the accumulation of eosinophils and IL-5 production are greatly reduced (Gonzalo *et al.*, 1996b; Hogan et al., 1997). These investigations support observations in this Chapter that IL-5 plays an important role in the mechanism of eotaxin-induced eosinophil trafficking during Th_2 type immune responses.

Similar to experiments in the skin (Chapter II), the i.v. administration of IL-5 enhanced the accumulation of eosinophils in the lungs in response to the localised production of IL-5 and eotaxin by the recombinant vaccinia viruses. The enhanced eosinophilic response in the airways in the presence of i.v. IL-5 may be due to the elevated circulating eosinophil pool. IL-5 may also prime the circulating eosinophils leading to the enhanced migration in response to chemotactic stimuli (IL-5 and eotaxin) in the lungs and promote the activation of adhesion systems in the vascular endothelium (Walsh *et al.*, 1990; Ebisawa *et al.*, 1994). Interestingly, the i.v. administration of IL-5 also enhanced the accumulation of eosinophils in the BALF in the presence of control virus. Viral infection may induce the production of RANTES by bronchial epithelial cells (Matsukura *et al.,* 1996; Becker *et al.,* 1997), which could then promote the accumulation of eosinophils in the airways.

Eosinophils are thought to be a primary effector cell in the induction of tissue damage and airways hyperreactivity in asthma and in animal models of allergic airway inflammation

(refer to introduction, section I.2) . However, the presence of eosinophils alone may not be enough to induce airways dysfunction and tissue damage. In guinea pigs, IL-5 induced pulmonary eosinophilia and inflammation, but not airways hyperreactivity (Lilly *et al.,* 1996). Upon activation, eosinophils have the ability to synthesise lipid mediators and oxygen radicals and may also release numerous cytotoxic cationic proteins. All of these inflammatory molecules have the potential to cause tissue damage and airways hyperreactivity (refer to introduction, section I.3). One of the cytotoxic cationic proteins in particular, MBP, has been implicated in the development airways hyperreactivity to β-methylcholine in both primates and rodents (Gundel *et al.,* 1991; Uchida *et al.,* 1993). Although VV-HA-IL-5 and VV-HA-eotaxin induced pronounced airways eosinophilia, no MBP was detected in the BALF, furthermore, these two cytokines did not promote airways hyperreactivity. These results demonstrate that the presence of IL-5 and eotaxin are not sufficient to induce eosinophil degranulation *in vivo* and airways hyperreactivity.

In contrast to these experiments, mice that were genetically altered for the over expression of IL-5 by airway epithelial cells, exhibited both pulmonary eosinophilia and airways hyperresponsiveness to β-methylcholine (Lee *et al.*, 1997). IL-5 over long periods of time induces the degranulation of eosinophils *in vitro* (Kita *et al.*, 1992) and this process may underlie this phenomena. Interestingly, airways hyperreactivity was induced in the presence of VV-HA-IL-5 and VV-HA-eotaxin following multiple exposures to aeroallergen. In contrast, VV-HA-TK (control virus) inoculation and aeroallergen exposure has no effect on airways reactivity. Antigen inhalation also resulted in the release of MBP from eosinophils in the presence of VV-HA-IL-5 and VV-HA-eotaxin. These results suggest that other factors are produced during antigen processing that are required for eosinophil degranulation and the induction of airways hyperreactivity.

MBP has been shown to mediate the induction of airways hyperreactivity in allergic airways inflammation in guinea pigs (Lefort *et al.,* 1996). Our results also show a correlation between the presence of MBP in the BALF and the induction of airways hyperreactivity; suggesting that the changes in airways reactivity that were seen in the presence of inhaled antigen were directly attributable to the action of eosinophil derived MBP. Moreover, it appears that the release of MBP by eosinophils is regulated by signals elicited during antigen processing.

Eosinophils express receptors for numerous classes of immunoglobulins, including IgG, IgA and IgE and each of these immunoglobulin types have been implicated in eosinophil degranulation (Looney *et al.,* 1986; Abu-Ghalez *et al.,* 1989; Hartnell *et al.,* 1992; Gounni *et al.,* 1994a, 1994b; Kaneko *et al.,* 1995). Thus, Ova-induced changes in airways reactivity following VV-HA-eotaxin and VV-HA-IL-5 inoculation may involve

the immunoglobulin dependent activation of eosinophils as Ova-specific IgG_1 was elevated in these mice.

Damage to pulmonary structure, including epithelial cell shedding occurs during allergic airways inflammation in mice (Foster *et al.,* 1996) and it appears likely that eosinophils mediate this process (Motojima *et al.*, 1989; Foster *et al.,* 1996). However, this particular action of the eosinophil may be secondary to the induction of airways hyperreactivity during allergy, since the induction of airways hyperreactivity by VV-HA-IL-5 and VV-HA-eotaxin following multiple exposures to aeroallergen did not induce gross morphological changes in pulmonary structure. These observations also suggest airways damage is not obligatory for the development of airways hyperreactivity.

The preferential localisation of eosinophils in regions that are directly below airways smooth muscle, but not between the bronchial epithelium and the basement membrane, suggests that eosinophils (or their products) may effect changes in airways reactivity by acting upon smooth muscle. Recently, it was demonstrated that MBP induces airways hyperreactivity by antagonising inhibitory M_2 muscarinic receptor during allergic airways inflammation in guinea pigs (Evans *et al.*, 1997). Thus, MBP may primarily induce airways hyperreactivity through this process; rather than as an effect of its cytotoxic properties upon the respiratory epithelium.

The recruitment of $CD4^+$ lymphocytes is a prominent feature associated with allergic eosinophilic inflammation (Bradley *et al.*, 1991; Bochner *et al.*, 1994). The accumulation of eosinophils and the induction of airways hyperreactivity during allergic airways inflammation has been shown to be dependent on the presence of mature $CD4^+$ T-lymphocytes (Gavette *et al.*, 1994; Gonzalo *et al.,* 1996). Furthermore, the development of late-phase eosinophilic response in murine model of cutaneous late-phase reaction (CLPR) is also inhibited by pretreatment with anti-CD4 mAb, but not by anti-CD8 mAb (Iwamoto *et al.,* 1992). However, previous studies could not define whether $CD4^+$ lymphocytes are required for eosinophil activation during allergic airways inflammation or for the recruitment of this cell.

Experiments in this Chapter demonstrate a direct dependence upon CD4 for the induction of eosinophil degranulation and airways hyperreactivity in the presence of IL-5, eotaxin and antigen processing. This process may involve the direct stimulation of eosinophil activation through $CD4^+$ lymphocyte-eosinophil interactions, possibly involving ICAM-1 (Hoire *et al.*, 1997) or MHC-II (Weller *et al.*, 1993). Eosinophil degranulation may also be stimulated by allergen specific immunoglobulins (IgG) whose production also appears to be dependent on the presence of CD4 in this model.

Further characterisation of mechanisms of eosinophil activation in the presence of IL-5, eotaxin and antigen may elucidate the role of the eosinophil in airways hyperreactivity in this system. The role of allergen specific immunoglobulins in this CD4-dependent phenomenon is unclear. Because the treatment of mice with anti-CD4 mAb abolishes the production of allergen specific IgG1 it is difficult to determine if the development of airways hyperreactivity is dependent on this molecule. It would be interesting to see if VV-HA-IL-5 and VV-HA-eotaxin can induce airways hyperreactivity in CD40 deficient mice [which do not develop Ova specific immunoglobulin IgG, IgA or IgE in a murine model of asthma, (Hogan *et al.*, 1996)] following multiple exposures to Ova aerosol.

In summary, the delivery of IL-5 and eotaxin together by recombinant vaccinia virus provides a useful tool for inducing pulmonary eosinophilia in mice in the absence of allergen or parasitic stimuli. Although IL-5 and eotaxin co-operatively induce pulmonary eosinophilia, these cytokines alone are not sufficient to cause airways hyperreactivity. However, these cytokines do promote airways hyperreactivity and eosinophil degranulation in the presence of antigen and this process is dependent on CD4. Furthermore, the localisation of eosinophils near airways smooth muscle and the correlation between the presence of MBP and the development of airways hyperreactivity suggests that eosinophils promote airways hyperreactivity by affecting airways smooth muscle function.

CHAPTER IV

DISSECTING THE REQUIREMENT OF IL-5 FOR EOSINOPHILIA

IV.1 INTRODUCTION.

In Chapters II and III it was demonstrated that IL-5 potently induced the accumulation of eosinophils in tissues and acted in synergy with the C-C-chemokines, eotaxin, MIP-1α and RANTES. Furthermore, the i.v. administration of IL-5 was shown to potentiate the tissue eosinophilia in response to these chemokines. The i.v. administration of IL-5 has also been shown to enhance the effectiveness of other eosinophil chemoattractants such as LTB_4 (Collins *et al.,* 1995) at sites of administration. IL-5 may promote eosinophil trafficking in response to chemoattractants by increasing the number of circulating eosinophils that are available to migrate into tissues and by priming eosinophils in the circulation leading to enhanced chemotactic responses. From studies in Chapter II it appears that eotaxin may also regulate the level of eosinophils in the circulation, although whether or not this is dependent on IL-5 has yet to be determined.

IL-5, in addition to acting as an eosinophil chemoattractant (Chapters II and III; Hakansson and Venge, 1994) and a regulator of eosinophil levels in the circulation (Kopf *et al.,* 1996), may also promote the adhesion of eosinophils to the vascular endothelium (Walsh *et al.,* 1990; Ebisawa *et al.,* 1994). Although it has been demonstrated that the responsiveness of eosinophils to chemoattractants are enhanced in the presence of IL-5 (chapters II and III, Coeffier *et al.*, 1994; Hakansson and Venge, 1994; Collins *et al.,* 1996), the role of basal production of this cytokine for eosinophil trafficking into tissues remains unclear.

In this chapter, IL-5 deficient mice [IL-$5^{-/-}$ (Kopf *et al.,* 1996)] were employed to dissect the requirement of IL-5 for blood and tissue eosinophilia at base line levels and in response to the eosinophil specific chemokine, eotaxin and in response to itself.

IV.2 MATERIALS AND METHODS.

IV.2.1 The role of IL-5 in eotaxin-induced eosinophil trafficking *in vivo.*

IV.2.1.1 *The effect of IL-5 or eotaxin on levels of circulating eosinophils in IL-5$^{-/-}$ mice.*

IL-5 (100-500 pmol/kg i.v.) and eotaxin (2.4 nmol/kg i.v.) were tested for their ability to induce blood eosinophilia in IL-5$^{-/-}$ mice (see section II.2.2). The effects of IL-5 (500 pmol/kg i.v.) or eotaxin (2.4 nmol/kg i.v.) on the bone marrow eosinophil levels of IL-5$^{-/-}$ mice were also determined (see section II.2.3), at the peak of the blood eosinophilia, induced by IL-5 (3 hours) or eotaxin (30 minutes). The effects of eotaxin and IL-5 on circulating and bone marrow levels of eosinophils in IL-5$^{+/+}$ mice were previously determined (see Chapter II).

IV.2.1.2 *Bioassay of the chemoattractant activity of IL-5 and eotaxin in IL-5$^{-/-}$ mice.*

IL-5 (10 pmol/skin site) and eotaxin (5 pmol/skin site) were analysed for their ability to induce the accumulation of eosinophils in the skin of IL-5$^{-/-}$ mice (see section II.2.4). IL-5$^{-/-}$ mice were injected i.v. with 500 pmol/kg of IL-5 (in place of 100 pmol/kg used in IL-5$^{+/+}$ mice as this concentration of IL-5 was required to elicit a pronounced elevation in circulating eosinophil levels). Similar experiments on IL-5$^{+/+}$ mice were previously performed (see Chapter II).

IV.2.1.3 *The production and purification of donor eosinophils.*

Donor eosinophils for homing experiments were obtained by sensitising mice (C57BL/6, 8-10 weeks old) by i.p. injection with 50 μg Ova and 1 mg Alhydrogel (CSL Ltd., Parkville, Australia) in 0.9% sterile saline on days 0 and 12. From day 24, sensitised mice received an i.p. injection of 50 μg OVA in 0.2 mls of 0.9% sterile saline, every second day for 8 days. Eosinophils were then washed from the peritoneal cavity with 3 x 5 mls of RPMI-1640 medium. In some experiments mice were injected with supernatants (0.5 mg protein/100 μl) obtained from cultured SF9 insect cells, instead of Ova, as this was found to induce a stronger i.p. eosinophilia. Eosinophils were purified by FACS using forward Vs side scatter and polarisation of light. The purity of the enriched population of eosinophils was > 98% as determined by differential staining with Giemsa-May-Grunwald. Purified eosinophils were diluted as required into RPMI-1640 and 100 μl injected into the tail vein of recipient mice. Greater than 97% of injected cells were determined as viable by trypan blue exclusion.

IV.2.1.4 *The role of basal production of IL-5 in the chemoattractant signal elicited by IL-5 and eotaxin for the recruitment of eosinophils into the skin.*

IL-$5^{-/-}$ and IL-$5^{+/+}$ mice were injected i.v. with 1 x 10^6 or 4 x 10^6 donor eosinophils (see section IV.2.1.3) and 10 minutes later peripheral blood samples were taken from the tail vein and analysed for eosinophil numbers using Discombe's method (see section II.2.2). The mice were then injected s.c. (see section II.2.4) with eotaxin [5.0 pmol/site (EC_{100})], IL-5 [10 pmol/site (EC_{100})] or control vehicle [100 µl HBSS/0.01% BSA (pH 7.4)]. After 2 hours the mice were sacrificed by CO_2 asphyxiation and the dorsal skin membranes excised onto a glass slide and prepared for differential cell counting (see section II.2.4).

IV.2.1.5 *The restoration of eosinophil homing in IL-$5^{-/-}$ mice by mini-asmotic pumps.*

IL-$5^{-/-}$ mice were anaesthetised by i.p. injection of 300 µl of a cocktail containing 60 mg/kg ketamine [Ketamil (Troy Laboratories Pty. Ltd., Smithfield, NSW., Australia)] and 8 mg/kg xylazine [Rompun (Bayer Australia Ltd., Pymble, NSW., Australia) and their ventral skin shaved and washed with hibitane. Mini-osmotic-pumps [M.O.P. (model 2001, Alzet Corporation, Palo Alto, USA)] containing IL-5 or control vehicle [10 mM PBS/0.1% BSA (pH 7.4)] were implanted i.p. under sterile conditions and the wound sealed. M.O.P. were calibrated to deliver control vehicle or 100 pmol/kg of IL-5 per hour for 7 days. After 72 hours mice were injected i.v. with IL-5 (500 pmol/kg) or control vehicle [100 µl of 10 mM PBS/0.1% BSA (pH 7.4)] at 0 and 30 minutes. Eotaxin (5.0 pmol/site), IL-5 (10 pmol/site) or control vehicle [100 µl of HBSS/0.01% BSA (pH 7.4)] was injected s.c. 30 minutes later. After 2 hours, the mice were sacrificed and the dorsal skin membrane excised for differential cell counts (see section II.2.4). Blood samples were taken before i.v. injection of IL-5 and at intervals of 30 minutes thereafter and eosinophils/ml of blood determined using Discombe's method (see section II.2.2).

IV.3 RESULTS.

IV.3.1 Eotaxin, but not IL-5, induces a rapid peripheral blood eosinophilia in IL-5$^{-/-}$ mice.

In IL-5$^{+/+}$ mice, the i.v. administration of 100 pmol/kg of IL-5 induced a rapid increase in the number of circulating eosinophils that peaked 30 minutes after administration (figure II.1a). In contrast, the i.v. administration of up to 5 times this concentration of IL-5 failed to induce a pronounced and rapid blood eosinophilia in IL-5$^{-/-}$ mice (figure IV.1a, results for < 500 pmol/kg i.v. IL-5 in IL-5$^{-/-}$ mice not shown, data included from figure II.1 for IL-5$^{+/+}$ mice that received 100 pmol/kg i.v. IL-5). Blood eosinophil levels peaked in IL-5$^{-/-}$ mice 3 hours post i.v. administration of 500 pmol/kg IL-5.

Similar to previous studies in IL-5$^{+/+}$ mice (Chapter II), the i.v. administration of IL-5 was associated with a reduction in the level of eosinophils in bone marrow (figure IV.2), when measured at the peak of the blood eosinophilia (30 minutes post administration of IL-5 in IL-5$^{+/+}$ mice and 3 hours in IL-5$^{-/-}$ mice). In contrast to IL-5, the i.v. administration of eotaxin (2.4 nmol/kg) induced a rapid and pronounced peripheral blood eosinophilia in IL-5$^{-/-}$ mice (figure IV.1b). The kinetics of the induction of peripheral blood eosinophilia by i.v. administration of eotaxin were similar between IL-5$^{+/+}$ and IL-5$^{-/-}$ mice (figure IV.1a). However, the peak of eosinophilia was diminished by approximately 50% in the IL-5$^{-/-}$ mice in comparison to that observed in IL-5$^{+/+}$ mice, at the same dose of eotaxin. As previously observed in IL-5$^{+/+}$ mice (section II.3.1), the increase in the number of eosinophils in the blood of IL-5$^{-/-}$ mice in response to eotaxin did not correlate with a significant reduction in the level of this cell in the bone marrow (figure IV.2).

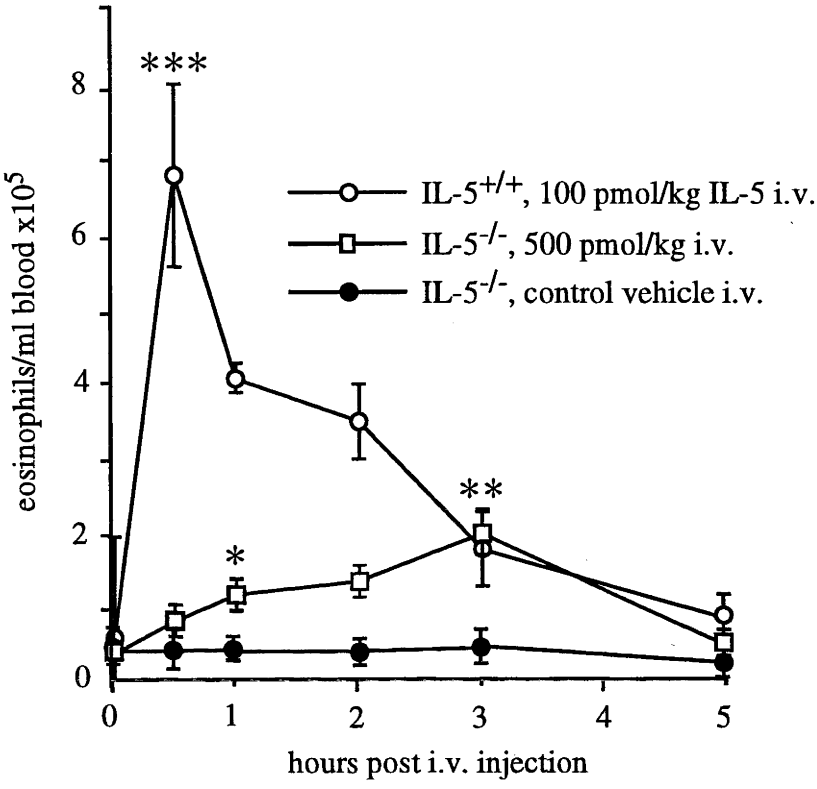
IV.3.2 In contrast to IL-5$^{+/+}$ mice, the subcutaneous administration of eotaxin and/or IL-5 to IL-5$^{-/-}$ mice fails to induce the accumulation of eosinophils in the skin.

In IL-5$^{-/-}$ mice, eotaxin (5.0 pmol/site), IL-5 (10 pmol/site) or a combination of both of these cytokines at the same site did not induce tissue eosinophilia at subcutaneous sites of administration, in contrast to IL-5$^{+/+}$ mice (figure IV.3, data for IL-5$^{+/+}$ mice included from figure II.4).

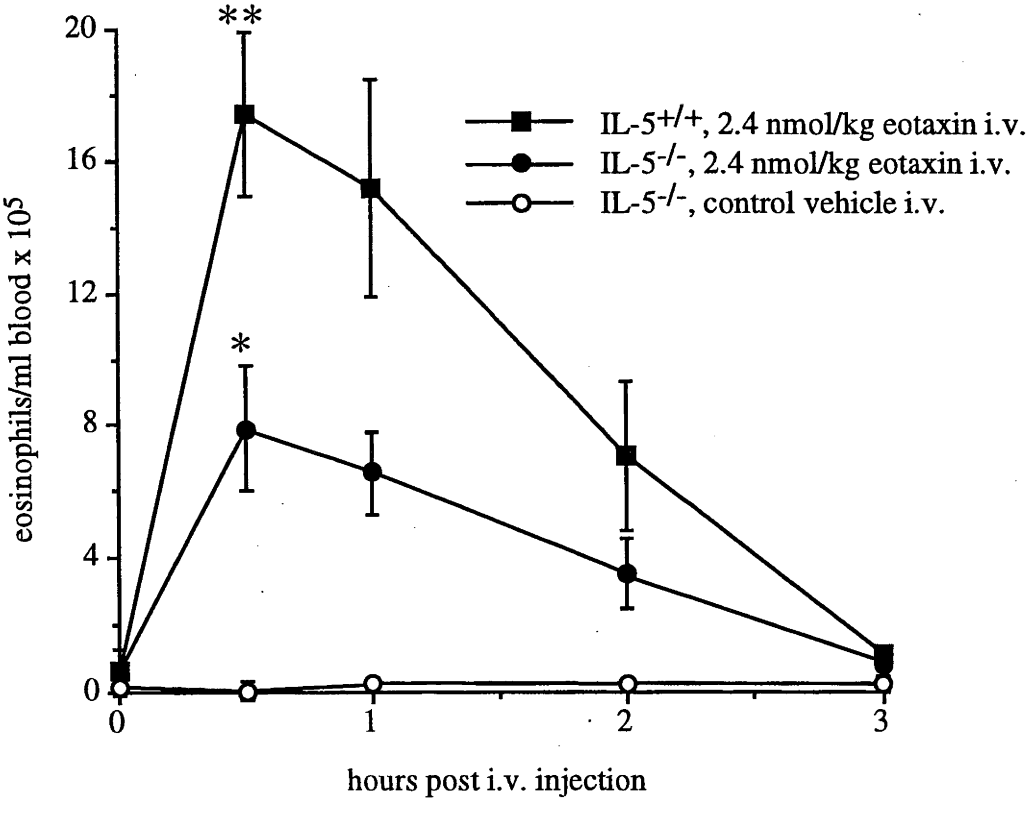
Figure IV.1 ***The effect of i.v. administration of IL-5 or eotaxin on circulating eosinophil numbers in IL-5$^{-/-}$ mice.***

Mice were injected i.v. with IL-5 (500 pmol/kg), eotaxin (2.4 nmol/kg) or control vehicle [(100 µl of 10 mM PBS/0.1% BSA (pH 7.4)]. Blood samples were taken before, at 30 minutes and hourly after i.v. injection of the cytokines or control vehicle for quantitation of circulating eosinophils. (a) IL-5 (500 pmol/kg) induced a transient increase in circulating eosinophil levels in IL-5$^{-/-}$ mice with slower kinetics and reduced potency in comparison to the response obtained in IL-5$^{+/+}$ mice at 100 pmol/kg i.v. (data included from figure II.1a). (b) Eotaxin (2.4 nmol/kg) induced a rapid increase in circulating eosinophil numbers in IL-5$^{-/-}$ mice, although levels were reduced by 50% in comparison to IL-5$^{+/+}$ mice (data included from figure II.1b). Results represent mean eosinophils/ml of blood ± SEM of groups of 5-6 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) * $P < 0.05$ and ** $P < 0.01$ compared with control vehicle at the same time point. *** $P < 0.001$ when compared with IL-5$^{-/-}$ mice at the same time point. (b) * $P < 0.001$ compared with control vehicle and ** $P <$ 0.01 when compared with IL-5$^{-/-}$ mice injected i.v. with eotaxin, at 30 minutes.

(a)


***
**
*
IL-5+/+, 100 pmol/kg IL-5 i.v.
IL-5-/-, 500 pmol/kg i.v.
IL-5-/-, control vehicle i.v.
eosinophils/ml blood x10^5
0
2
4
6
8
0
1
2
3
4
5
hours post i.v. injection

(b)


**
*
IL-5+/+, 2.4 nmol/kg eotaxin i.v.
IL-5-/-, 2.4 nmol/kg eotaxin i.v.
IL-5-/-, control vehicle i.v.
eosinophils/ml blood x 10^5
0
4
8
12
16
20
0
1
2
3
hours post i.v. injection

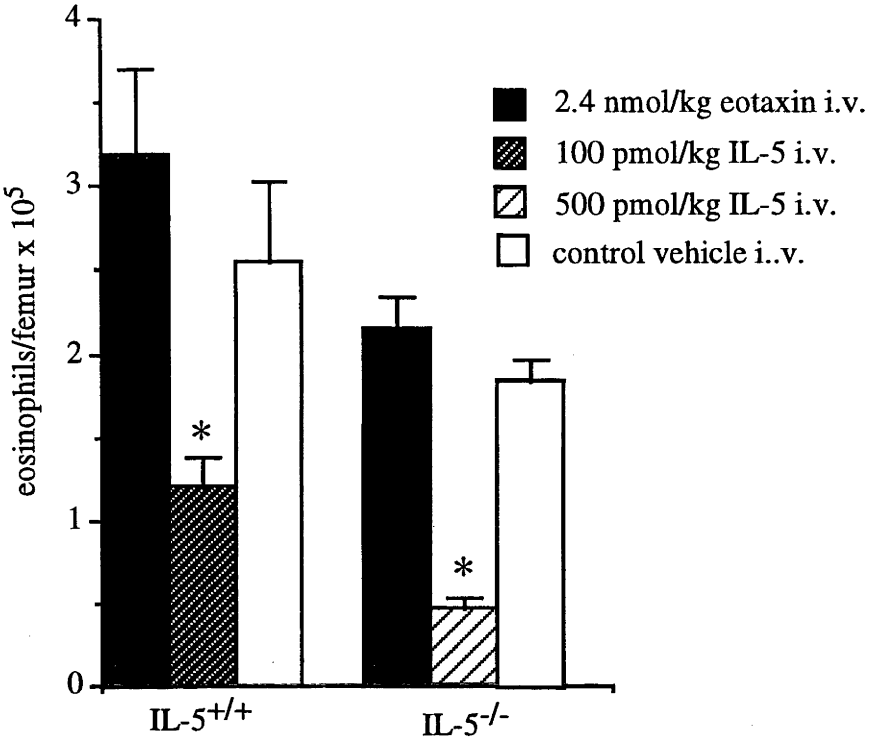


Figure IV.2 ***Eosinophil numbers in the bone marrow of IL-5-/- mice in the presence of i.v. IL-5 or eotaxin.***

IL-5 (100 pmol/kg and 500 pmol/kg, for IL-$5^{+/+}$ and IL-$5^{-/-}$ mice, respectively), eotaxin (2.4 nmol/kg) or control vehicle [100 µl of 10 mM PBS/0.1% BSA (pH 7.4)] were injected i.v. into mice. Femurs were removed at the peak of the blood eosinophilic response [30 minutes in IL-$5^{+/+}$ mice (data included from figure II.2) and 3 hours in IL-$5^{-/-}$ mice with i.v. IL-5 and 30 minutes for all mice with i.v. eotaxin (data included for IL-$5^{+/+}$ mice from figure II.2)] and the bone marrow cavity lavaged with 2 mls of HBSS. The number of cells/ml of lavage fluid was determined and 2 x 10^5 cells were cytocentrifuged and stained with Giemsa-May-Grunwald for differential cell counting. Results represent mean bone marrow eosinophils ± SEM of groups of 4 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. $^*P < 0.05$ compared with i.v. control vehicle.

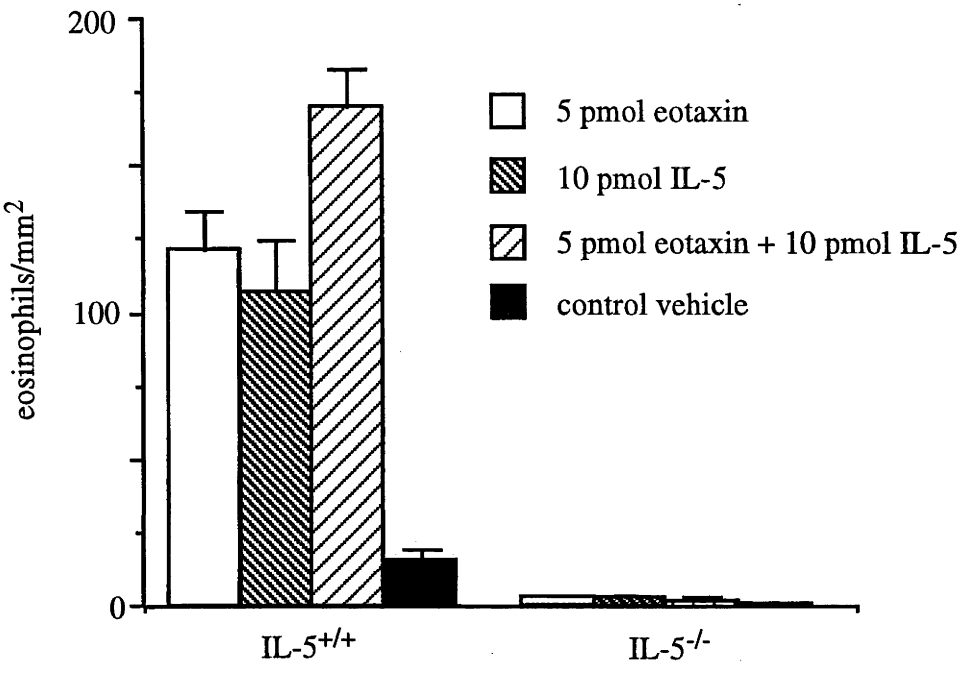


Figure IV.3 ***Eotaxin and/or IL-5 do not induce the accumulation of eosinophils into the skin when administered s.c. to IL-5$^{-/-}$ mice.***

IL-5$^{-/-}$ mice were injected i.v. with 500 pmol/kg of IL-5 and 1 hour later were given a s.c. injection of eotaxin (5 pmol/skin site), IL-5 (10 pmol/skin site), a combination of both cytokines or control vehicle [100 µl of 10 mM PBS/0.1% BSA (pH 7.4)]. Eosinophil accumulation in skin membranes was assessed at 2 hours post s.c. injection. Results represent mean eosinophils/mm^2 ± SEM of groups of 6 mice. One dorsal membrane preparation was excised from each animal and prepared for differential cell counting. Ten fields per preparation were counted for eosinophil infiltration and the mean obtained. For comparative purposes, data from previous experiments in IL-5$^{+/+}$ mice under similar conditions (figure II.4a) has also been included. IL-5$^{+/+}$ mice were treated similar to IL-5$^{-/-}$ mice except they received an i.v. injection of 100 pmol/kg (see section II.2.4) in place of 500 pmol/kg of IL-5 i.v. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. No significant levels of eosinophils were detected in the skin of IL-5$^{-/-}$ mice under any treatment.

IV.3.3 The adoptive transfer of eosinophils enhances the accumulation of eosinophils at skin sites of eotaxin or IL-5 administration in IL-$5^{+/+}$, but not IL-$5^{-/-}$, mice.

To determine whether eotaxin-induced tissue eosinophilia was solely dependent on IL-5 or on the available pool of circulating eosinophils and to ascertain the role of these cytokines in eosinophil homing, donor eosinophils were injected i.v. into IL-$5^{+/+}$ and IL-$5^{-/-}$ mice (figure IV.4). The i.v. adoptive transfer of 4 x 10^6 donor eosinophils into IL-$5^{+/+}$ and IL-$5^{-/-}$ mice (figure IV.4a) established blood eosinophil levels in these animals equivalent to those which were induced by i.v. IL-5 (100 pmol/kg) in IL-$5^{+/+}$ mice [figure IV.1a, ie. at levels that promoted enhanced the accumulation of eosinophils in tissues in response to eotaxin (figure II.3a) or IL-5 (figure II.3c)]. Eosinophil levels in the blood following the adoptive transfer of 4 x 10^6 eosinophils were also similar to that observed during allergic responses in mice (Foster *et al.*, 1996). The adoptive transfer of eosinophils (1 x 10^6 donor cells) to IL-$5^{+/+}$ mice significantly enhanced the accumulation of eosinophils in the skin in response to eotaxin (5.0 pmol/site) (figure IV.4b) or IL-5 (10 pmol/site) (figure IV.4c). Responses to eotaxin were significantly amplified by transferring 4 x 10^6 donor cells to IL-$5^{+/+}$ mice (figure IV.4b). In contrast, injection of up to 4 x 10^6 donor eosinophils into IL-$5^{-/-}$ mice did not promote eosinophil recruitment to sites of eotaxin (5.0 pmol/site) (figure IV.4b). The injection of 1 x 10^6 donor eosinophils into IL-$5^{-/-}$ mice also failed to promote the accumulation of eosinophils at sites of IL-5 (10 pmol/site) (figure IV.4c) administration.

IV.3.4 Restoration of eotaxin- and IL-5- induced tissue eosinophilia and eosinophil homing in IL-$5^{-/-}$ mice.

The central role of IL-5 in eotaxin- or IL-5- induced eosinophil recruitment was confirmed in IL-$5^{-/-}$ mice pretreated with IL-5 i.p. for 72 hours using M.O.P. The continuous i.p. administration of IL-5 by M.O.P. in the pretreatment did not induce blood eosinophilia (figure IV.5a). However, subsequent administrations of IL-5 (500 pmol/kg i.v.) induced a rapid blood eosinophilia (figure IV.5a) equivalent to that induced by IL-5 (100 pmol/kg i.v.) in IL-$5^{+/+}$ mice (figure II.1a). Moreover, this level of eosinophils in the circulation of IL-$5^{+/+}$ mice promoted enhanced eosinophilic responses to eotaxin (figure II.3a) or IL-5 (figure II.3c) in tissues. The ability of eotaxin (5.0 pmol/site) and IL-5 (10 pmol/site) to induce the recruitment of eosinophils into tissues was also restored (figure IV.5b).

Figure IV.4 ***The role of IL-5 in eosinophil homing to the skin.***

(a) Eosinophil levels in the blood after adoptive transfer of eosinophils to IL-$5^{+/+}$ and IL-$5^{-/-}$ mice. Mice were injected i.v. with donor eosinophils (1 x 10^6 or 4 x 10^6 cells into mice receiving s.c. eotaxin or 1 x 10^6 cells into mice receiving s.c. IL-5) or control vehicle [100 µl of 10 mM PBS (pH 7.4)] and 10 minutes later peripheral blood samples were taken from the tail vein. Circulating eosinophil numbers in IL-$5^{+/+}$ mice given 4 x 10^6 donor eosinophils i.v. and in IL-$5^{-/-}$ mice given 1 x 10^6 or 4 x 10^6 donor eosinophils, were significantly elevated relative to circulating eosinophil levels in mice that did not receive i.v. donor eosinophils. (b) Mice injected s.c. with eotaxin (5.0 pmol/site) or control vehicle [100 µl HBSS/0.01% BSA (pH 7.4)] immediately following the i.v adoptive transfer of eosinophils. After 2 hours the mice were sacrificed and the dorsal skin membrane excised and prepared for differential cell counting. Donor eosinophils significantly enhanced the accumulation of eosinophils into the skin in response to eotaxin (5.0 pmol/site) in IL-$5^{+/+}$ mice. In contrast, the adoptive transfer of eosinophils to IL-$5^{-/-}$ mice did not promote eosinophil recruitment to sites of eotaxin (5.0 pmol/site) exposure. (c) Mice were treated as for (b) above with the following exceptions. IL-$5^{+/+}$ were injected i.v. with 1 x 10^6 donor eosinophils or control vehicle [100 µl of 10 mM PBS (pH 7.4)] and IL-5 (10 pmol/site) was injected s.c. This level of donor eosinophils was used since it promoted enhanced the accumulation of eosinophils at skin sites of eotaxin or IL-5 administration in IL-$5^{+/+}$ mice. Results represent (a) mean eosinophils/ml of blood ± SEM and (b and c) mean eosinophils/mm^2 ± SEM. Ten fields (200 x magnification) were counted from each membrane preparation. Data for all experiments was obtained from groups of 5-6 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) * $P < 0.01$ and ** $P < 0.001$ when compared to i.v. control vehicle. (b) * $P < 0.01$ and ** $P < 0.001$ when compared with 5.0 pmol eotaxin/skin site with i.v. control vehicle. (c) * $P < 0.01$ when compared with 10 pmol IL-5/skin site with i.v. control vehicle.

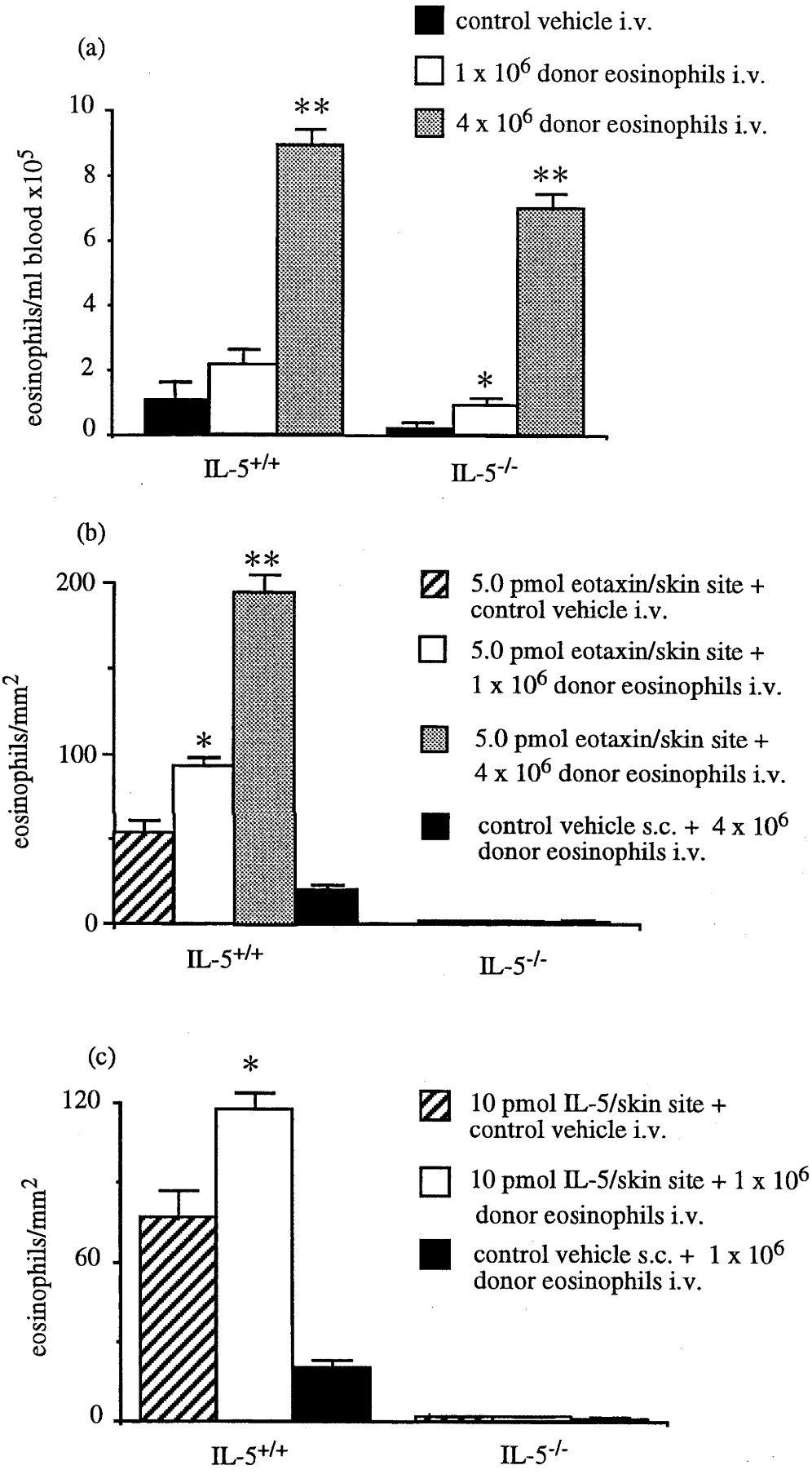
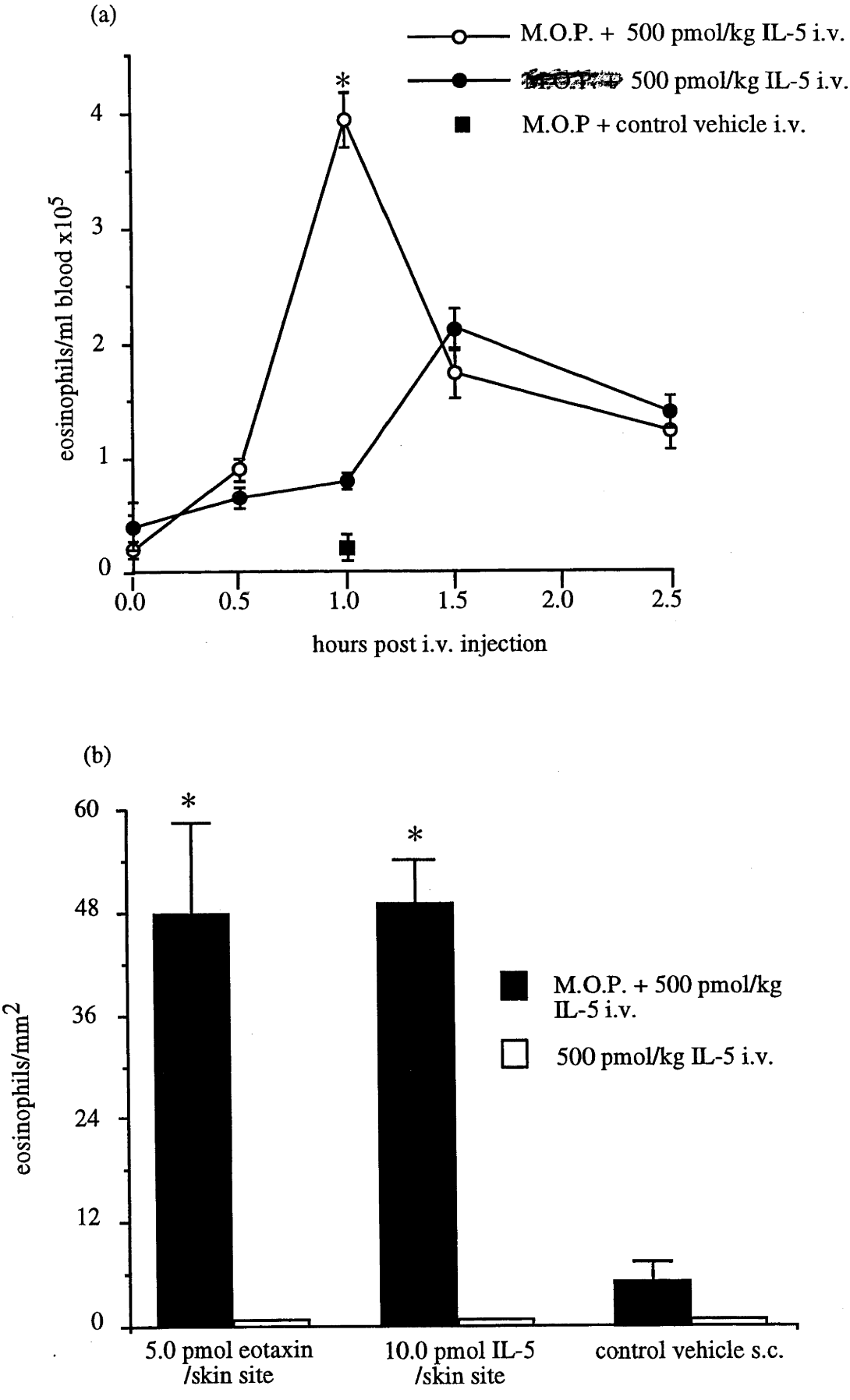

(a)
control vehicle i.v.
1 x 10^6 donor eosinophils i.v.
4 x 10^6 donor eosinophils i.v.
eosinophils/ml blood x10^5
10
8
6
4
2
0
**
**
*
IL-5+/+
IL-5-/-
(b)
eosinophils/mm^2
200
100
0
**
*
5.0 pmol eotaxin/skin site + control vehicle i.v.
5.0 pmol eotaxin/skin site + 1 x 10^6 donor eosinophils i.v.
5.0 pmol eotaxin/skin site + 4 x 10^6 donor eosinophils i.v.
control vehicle s.c. + 4 x 10^6 donor eosinophils i.v.
IL-5+/+
IL-5-/-
(c)
eosinophils/mm^2
120
60
0
*
10 pmol IL-5/skin site + control vehicle i.v.
10 pmol IL-5/skin site + 1 x 10^6 donor eosinophils i.v.
control vehicle s.c. + 1 x 10^6 donor eosinophils i.v.
IL-5+/+
IL-5-/-

Figure IV.5 ***Restoration of eotaxin- and IL-5- induced tissue eosinophilia and eosinophil homing in IL-5$^{-/-}$ mice using mini-osmotic pumps.***

(a) IL-5$^{-/-}$ mice were pretreated for 72 hours with i.p. IL-5 using mini-osmotic-pumps (M.O.P.). Blood eosinophilia was induced in these mice by two i.v. injections of IL-5 (500 pmol/kg) at 0 and 30 minutes. Intravenous injection of IL-5 in mice which were not pretreated for 72 hours with i.p. IL-5 failed to induce a pronounced blood eosinophilia. Blood eosinophilia was not induced by i.v. injection of control vehicle [100 µl of 10 mM PBS/0.1% BSA (pH 7.4)] in IL-5 (M.O.P.) pretreated mice. Blood samples were taken before i.v. injection of IL-5 and at intervals of 30 minutes thereafter. The resulting eosinophilia at 30 minutes after the second i.v. injection of IL-5 in IL-5 (MOP) pretreated mice was equivalent to that induced by this cytokine (figure IV.1a) at the time of subcutaneous administration of eotaxin (figure II.3a) or IL-5 (figure II.3c) in IL-5$^{+/+}$ mice. (b) Eotaxin (5.0 pmol/site), IL-5 (10 pmol/site) or control vehicle [HBSS/0.01% BSA (pH 7.4)] were injected subcutaneously 30 minutes after the second i.v. injection of IL-5 or control vehicle and 2 hours later the mice were sacrificed and the dorsal skin membrane excised for differential cell counts. Eotaxin- and IL-5- induced recruitment of eosinophils to sites of exposure was only restored in IL-5$^{-/-}$ mice that were pretreated i.p. with IL-5 for 72 hours. Results represent (a) mean eosinophils/ml of blood ± SEM of groups of four mice and (b) mean eosinophils/mm^2 ± SEM of groups of four mice. Ten fields were counted from each membrane preparation. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) * $P < 0.001$ when compared with i.v. IL-5 in the absence of IL-5 pretreatment by M.O.P. and (b) * $P <$ 0.001 when compared with i.v. IL-5 in the absence of IL-5 pretreatment by M.O.P.


(a)
M.O.P. + 500 pmol/kg IL-5 i.v.
500 pmol/kg IL-5 i.v.
M.O.P + control vehicle i.v.
eosinophils/ml blood x10^5
0
1
2
3
4
*
0.0
0.5
1.0
1.5
2.0
2.5
hours post i.v. injection
(b)
eosinophils/mm^2
0
12
24
36
48
60
*
*
M.O.P. + 500 pmol/kg IL-5 i.v.
500 pmol/kg IL-5 i.v.
5.0 pmol eotaxin /skin site
10.0 pmol IL-5 /skin site
control vehicle s.c.

IV.3 DISCUSSION.

IL-5 regulates basal circulating eosinophil levels (Kopf, *et al.*, 1996) and this cytokine promotes blood eosinophilia (Dent, *et al.*, 1990). The i.v. administration of IL-5 (100 pmol/kg) induced a rapid and pronounced blood eosinophilia in IL-$5^{+/+}$ mice (Chapter II). In contrast, IL-5 at doses up to 5 times this concentration failed to induce a rapid and pronounced blood eosinophilia in IL-$5^{-/-}$ mice. Data in IL-$5^{-/-}$ mice suggests that the mechanism involved in the rapid induction of blood eosinophilia and the maintenance of basal circulating eosinophil levels requires the presence of this cytokine. Interestingly, IL-$5^{-/-}$ mice developed a delayed blood eosinophilia following the i.v. administration of IL-5 and this response correlated with a reduction in bone marrow levels of this cell.. - Thus, the ability of IL-5 to induce peripheral blood eosinophilia in IL-$5^{-/-}$ mice, although delayed when compared to its action in IL-$5^{+/+}$ mice, appears to involve at least partially the mobilisation of eosinophils from the bone marrow. The inability of i.v. IL-5 to induce a rapid blood eosinophilia in IL-$5^{-/-}$ mice may not soley reflect the reduced capacity of this cytokine to mobilise bone marrow eosinophils into the circulation. IL-$5^{-/-}$ mice have reduced basal levels of eosinophils in the blood which may affect the number of eosinophils migrating through tissues (Kopf *et al.*, 1996) and thus, eosinophils migrating from the bone marrow compartment may be rapidly sequestered from the circulation into tissues.

The ability of eotaxin to induce a rapid increase in eosinophil levels in the blood of IL-$5^{-/-}$ mice and the demonstration that eotaxin does not mobilise eosinophils from the bone marrow suggests that this cytokine regulates eosinophilia via different mechanisms to that of IL-5. Furthermore, eotaxin induces blood eosinophilia independently of IL-5 and may recruit tissue eosinophils into the circulation. Although eotaxin-induced blood eosinophilia was significantly reduced in IL-$5^{-/-}$ mice, in comparison to IL-$5^{+/+}$ mice, this does not necessarily indicate a direct role for IL-5 in this process. The reduced eosinophilic response to eotaxin may also reflect the number of eosinophils available to be recruited from tissues into the blood of IL-$5^{-/-}$ mice. Collectively, these experiments demonstrate a novel mechanism for the induction of blood eosinophilia that is regulated independently of IL-5. Eotaxin expression is upregulated in endothelial cells by IFNγ (Rothenberg *et al.*, 1995a), providing a mechanism for its release into the blood. Eotaxin is expressed in tissues quickly following antigen provocation and elevated levels of this cytokine in the blood may assist a rapid eosinophilic response in inflamed tissues by amplifying the circulating eosinophil pool. This eotaxin regulation would provide a mechanism to rapidly elevate the available pool of blood eosinophils to be recruited to sites of allergen provocation. During later stages of the allergic reaction, IL-5, produced by infiltrating Th_2 lymphocytes may then promote the eosinophilic response by inducing and maintaining blood eosinophilia (Dent *et al.*, 1990; Foster, *et al.*, 1996).

Although numerous molecules induce eosinophil chemotaxis and/or the accumulation of eosinophils in tissues at sites of instillation, only eotaxin and IL-5 appear to specifically control the migration of this leukocyte (Wardlaw *et al.*, 1994; Rothenberg *et al.*, 1996; Chapter II). Furthermore, IL-5 potently amplifies the eotaxin signal (and that of other C-C chemokines) (Collins *et al.*, 1995; Rothenberg *et al.*, 1996; Chapter II). However, the requirement for IL-5 in the trafficking of eosinophils in tissues in response to this chemoattractant is unknown.

Neither s.c. eotaxin nor IL-5 induced the accumulation of eosinophils in the skin of IL-5$^{-/-}$ mice (figure IV.3). This was in contrast to the action of these two cytokines in IL-5$^{+/+}$ mice, where they are potent eosinophil chemoattractants. In these experiments, all animals received an i.v. injection of IL-5 (500 pmol/kg for IL-5$^{-/-}$ and 100 pmol/kg for IL-5$^{+/+}$ mice) as this enhanced the circulating eosinophil pool and was previously shown to enhance the eosinophilic response to chemoattractants in tissues in wild type mice (see section II.3.2). The inability of IL-5$^{-/-}$ mice to recruit eosinophils into tissues at sites of eotaxin and/or IL-5 instillation did not reflect the reduced circulating level of eosinophils in these animals since intravenous administration of IL-5 restored circulating eosinophil numbers to levels that are sufficient for basal eosinophilic responses in the tissues of IL-5$^{+/+}$ mice. Furthermore, at times of peak eosinophil accumulation in the skin (3 hours post i.v. administration of IL-5), circulating eosinophil levels were the same in IL-5$^{-/-}$ and IL-5$^{+/+}$ mice. This indicates that equivalent amounts of this leukocyte were available for trafficking into tissues in both groups at this time. The adoptive transfer of eosinophils also enhanced the accumulation of eosinophils into the skin in response to eotaxin and IL-5 in IL-5$^{+/+}$ mice, but not in IL-5$^{-/-}$ mice. These results further demonstrate that increased levels of circulating eosinophils alone are not sufficient to promote the enhanced recruitment of eosinophils to sites of eotaxin or IL-5 administration in IL-5$^{-/-}$ mice. Furthermore, the adoptive transfer experiments suggest that the failure of IL-5$^{-/-}$ mice to recruit eosinophils into tissues is due to impaired eosinophil homing mechanisms at the tissue level.

The i.p. pretreatment of IL-5$^{-/-}$ mice with IL-5 by M.O.P. restored the ability of these mice to accumulate eosinophils at subcutaneous sites of eotaxin and IL-5 administration and thus, establishes an essential role for IL-5 in the chemoattractant signal elicited by these cytokines. This result also suggests that basal levels of IL-5 may play a critical role in regulating eosinophil homing by other molecules. IL-5 may promote/regulate basal eosinophil migration in tissues by activating adhesion systems on the vascular endothelium (Walsh *et al.*, 1990; Ebisawa *et al.*, 1994). Future studies, specifically looking at the local expression and/or activation of different adhesion systems at sites of s.c eotaxin administration in IL-5$^{+/+}$ mice, IL-5$^{-/-}$ mice and IL-5$^{-/-}$ mice pretreated i.p. with IL-5 by M.O.P. may elucidate the mechanism behind the requirement for IL-5 in

eosinophils trafficking in response to chemoattractants. Collectively these results demonstrate an essential role for IL-5 in eosinophil homing and migration into tissues in response to specific chemoattractant stimuli.

CHAPTER V

THE ROLE OF INTERLEUKIN-5 IN EOSINOPHIL TRAFFICKING AND FUNCTION DURING ALLERGY

V.1 INTRODUCTION.

Numerous studies in animal models of allergic inflammation have shown a key role for IL-5 in regulating eosinophil trafficking and in the development of disease (Iwamoto *et al.*, 1992; Nakajima *et al.*, 1992; Bozza *et al.*, 1994; Foster *et al.*, 1996; Lee *et al.*, 1997). However, the action of IL-5 in these experimental systems remains unclear.

Iwamoto *et al.*, (1992) have reported that anti-IL-5 mAb inhibits the second phase (but not the first phase) of the accumulation of eosinophils at sites of allergen-induced cutaneous late-phase reactions in mice. These studies suggested that IL-5 may act as an eosinophil chemoattractant, however, the action of IL-5 in these experiments was not defined. IL-5 has a multitude of effects on eosinophils. This cytokine not only acts as a chemoattractant for eosinophils but also primes eosinophils for function, regulates the level of eosinophils levels in the circulation and can activate adhesion mechanisms utilised by the cell (Walsh *et al.*, 1990; Ebisawa *et al.*, 1994; Hakansson and Venge, 1994; Kopf *et al.*, 1996). Thus, anti-IL-5 mAb could inhibit any one or more of these processes.

IL-5$^{-/-}$ mice do not develop a pronounced pulmonary eosinophilia during allergic airways inflammation, in contrast to wild types. ~~[In addition, IL-5$^{-/-}$ mice do not exhibit gross airways damage or pronounced airways hyperreactivity that normally follows exposure to aeroallergen]~~ (Foster *et al.*, 1996). In comparison to the wild type, IL-5$^{-/-}$ mice also have lower circulating levels of eosinophils and their ability to mount a blood eosinophilia in response to antigen provocation is markedly impaired (Foster *et al.*, 1996). Although these data indicate an important role for IL-5 in regulating eosinophilia they do not dissect the role of this cytokine in individual steps regulating eosinophil migration. The failure of IL-5$^{-/-}$ mice to develop tissue eosinophilia in response to antigen-stimulation may reflect the lower circulating pool rather than a direct involvement of IL-5 in regulating eosinophil homing, adhesion or chemotaxis.

The role of IL-5 in the induction of airways hyperreactivity during allergic airways inflammation is also unclear. Sensitised IL-5$^{-/-}$ mice do not develop airways damage or hyperreactivity following exposure to aeroallergen (Foster *et al.*, 1996). ~~In addition, these mice do not develop a pronounced pulmonary eosinophilia and thus,~~ It remains unclear whether or not it is the eosinophil or IL-5 (or both) that are required for disease. Of all inflammatory cytokines, IL-5 is the most intimately associated with eosinophil function and therefore, it has been difficult to separate the roles of IL-5 and eosinophils in the pathogenesis of allergic airways disease.

Mice that were genetically altered for the specific over production of IL-5 in the airway epithelial clara cell (Lee *et al.*, 1997) displayed airways hyperreactivity to inhaled

methacholine. These mice also exhibited a pulmonary eosinophilia and morphological changes gnomonic of asthma. Therefore, similar to studies in IL-5$^{-/-}$ mice, it was difficult to separate the role of IL-5 from that of the eosinophil in the induction of airways hyperreactivity in these mice.

In this Chapter, by using IL-5$^{-/-}$ mice, we have studied the individual roles of this cytokine in eosinophil recruitment during allergy in the lung and skin. Furthermore, by selectively amplifying the eosinophilic response in absence of IL-5 using recombinant eotaxin, the role of this cell and IL-5 in the development of airways hyperreactivity in mice, following aeroallergen provocation, was examined.

V.2 MATERIAL AND METHODS.

V.2.1 The role of IL-5 in eosinophil trafficking during allergy.

V.2.1.1 *The induction of cutaneous late-phase reaction in mice.*

Cutaneous late-phase reaction (CLPR) was induced in mice using methods similar to those previously described (Iwamoto *et al.,* 1992). Briefly, male C57BL6 mice, 6-8 weeks of age were immunised by i.p. injection of 50 µg Ova and 1 mg Alhydrogel (CSL Ltd., Parkville, Australia) in 0.2 mls of sterile 0.9% saline on days 0 and 12. Non-sensitised mice received 1 mg Alhydrogel in sterile saline only. On day 24 the mice received a s.c. co-injection of 50 µg Ova in 100 µl of sterile 0.9% saline plus 900 µl of air into the upper dorsal flank. Control animals received saline and air only. At 0, 3, 6, 9, 12, 24, 48 and 72 hours after subcutaneous injections a blood sample was taken from the tail vein and the animals sacrificed by CO_2 asphyxiation. The dorsal skin membrane was then excised and prepared for differential cell counting (see section II.2.4). Eosinophils/ml of blood were determined using Discombe's methods (see section II.2.2).

V.2.1.2 *Determining the role of IL-5 in eosinophils accumulation during cutaneous late-phase reaction.*

CLPR was induced in IL-$5^{+/+}$ and IL-$5^{-/-}$ mice (male, 6-8 weeks of age) (see section V.2.1.1). At 6 and 24 hours post s.c. injection the mice were sacrificed and the dorsal skin membrane excised and prepared for differential cell counting (see section II.2.4). Blood samples were taken at 0, 6 and 24 hours post s.c. injection and ~~eosinophils/ml of~~ the number of eosinophils per ml of blood were quantified using Discombe's method (see section II.2.2).

V.2.1.3 *Determining the role of IL-5 in eosinophils homing during cutaneous late-phase reaction.*

Donor eosinophils were produced in IL-$5^{+/+}$ mice by twice weekly i.p. injections of 100 µg Polymyxin B sulphate (SIGMA Chemical Co., St Louis, MO., USA.) in 0.2 mls of sterile 0.9% saline for a period of 6-8 weeks (Pincus, 1978). Eosinophils were collected from the peritoneal cavity and purified by FACS (see section IV.2.1.3). Purified eosinophils were labelled with the non-toxic fluorescent nuclear stain, H33342 (SIGMA Chemical Co., St Louis, MO., USA.) using the methods of Lyons and Parish (1994). Briefly, FACS-purified eosinophils (see section IV.2.1.3) were pelleted by centrifugation (400 x g for 5 minutes, 4°C) and resuspended at 1 x 10^7 cells/ml in RPMI-1640. One microlitre of H33342 (5 mM in DMSO) was then added to give a final concentration of 5 µM H33342 and the eosinophils incubated at 37°C for 10 minutes in the absence of light.

After washing twice in ice cold serum free RPMI-1640 medium, eosinophil viability was assessed by trypan blue exclusion (> 95% viable).

CLPR was induced in IL-$5^{+/+}$ and IL-$5^{-/-}$ mice (see section V.2.1.1). At 4 or 22 hours post s.c. injection the mice were given an i.v. injection of 1 x 10^6 H33342-labelled eosinophils in 100 µl of RPMI-1640. The adoptive transfers were confirmed by taking a blood sample 10 minutes after i.v. injection, via the tail vein and fluorescent cells/ml of blood quantified. Briefly, following red cell lysis, fluorescent cells/ml blood were quantified using fluorescence microscopy (Ex 355 nm, Em 460 nm) and a haemocytometer. The mice were sacrificed 2 hours after adoptive transfer (at 6 or 24 hours after induction of CLPR) and the dorsal skin membrane of each animal was excised onto a glass slide. Membrane sections were viewed immediately by fluorescence microscopy (Ex 355 nm, Em 460 nm) and fluorescent cells/mm^2 were determined by counting 10 fields (200 x magnification). Representative sections were photographed using Tri-X pan-100 black and white film (Eastman Kodak Company, Rochester, NY., USA.).

V.2.1.4 *Induction of allergic airways inflammation.*

IL-$5^{+/+}$ and IL-$5^{-/-}$ mice (male, 8-10 weeks of age) were sensitised to Ova by i.p. injection on days 0 and 12 (see section V.2.1.1). Non-sensitised mice received 1 mg of Alhydrogel in saline only. On day 24, the sensitised mice were exposed to an aerosol of Ova (10 mg/ml) in 0.9% saline for 1 hour and the controls were exposed to saline aerosol. The blood and BALF levels of eosinophils were analysed at 0, 3, 6, 9,12, 15, 18, 21, 24, 36 and 48 hours following the aerosol exposure (see sections II.2.2 and III.2.9). In other experiments, some mice were exposed to an aerosol of Ova (10 mg/ml in saline) on day 24 for 3 x 30 minutes instead of 1 hour, with 30 minutes rest between the exposures. These mice were further exposed to Ova aerosol every second day thereafter for a total of 6 days. Airways hyperreactivity to i.v. β-methylcholine was analysed (see section III.2.11) on days 23, 25, 27, 29 and 31. Immediately following the analysis of airways hyperreactivity the mice were sacrificed by cervical dislocation and the eosinophils/ml of BALF were then quantified (see section III.2.9). Blood smears were prepared on glass slides from blood samples taken prior to the analysis of airways hyperreactivity and the levels of the different types of circulating leukocytes were quantified after staining with May-Grunwald-Giemsa solution. Two to three hundred leukocytes were routinely counted on each slide and cell types were identified by morphological criteria.

V.2.1.5 *Determining the role of IL-5 in eosinophils homing to the allergic lungs of mice.*

Eosinophils were produced, purified and then labelled with the non-toxic fluorescent nuclear stain H33342 (see section V.2.1.3). IL-$5^{+/+}$ and IL-$5^{-/-}$ mice were sensitised to Ova by i.p. injection on days 0 and 12 (see section V.2.1.1) and on day 24 the mice were exposed to an aerosol of Ova (10 mg/ml) in 0.9% saline for 1 hour. Non-sensitised control groups were exposed to an aerosol of saline instead of Ova. Six hours later all groups of mice received an i.v. adoptive transfer of 5 x 10^6 H33342-labelled eosinophils. Adoptive transfers were confirmed by taking a blood sample 10 minutes after transfer and the fluorescent cells/ml of blood quantified (see section V.2.1.2). The levels of H33342-labelled eosinophils in the airways were determined 24 hours after aerosol exposure by lavaging the lungs with 2 x 1 ml of HBSS and counting fluorescent eosinophil/ml BALF using a haemocytometer and fluorescence microscopy (Ex 355 nm, Em 460 nm).

These homing experiments were performed during a mild allergen-induced pulmonary inflammation (after 1 aerosol) to keep the total and individual leukocyte populations in the lungs of IL-$5^{+/+}$ and IL-$5^{-/-}$ mice as similar as possible. Chronic allergic airways inflammation in mice, induced by the repeated exposures of sensitised mice to aerosolized Ova, leads to a more pronounced elevation in leukocyte populations, specifically lymphocytes and eosinophils in the BALF of IL-$5^{+/+}$ mice compared with the BALF of IL-$5^{-/-}$ mice (Foster *et al.,* 1996) and this would have complicated the interpretation of experimental results.

V.2.1.6 *The enhancement of pulmonary eosinophilia and airways hyperreactivity in IL-$5^{-/-}$ mice during allergic airways inflammation by i.v. adoptive transfer of eosinophils or i.v. eotaxin.*

IL-$5^{-/-}$ mice (male, 8-10 weeks of age) were sensitised to Ova by i.p. injection on days 0 and 12 (see section V.2.1.1). On days 24, 26, 27 and 28, the mice were exposed to an aerosol of Ova (10 mg/ml) in 0.9% saline for 3 x 30 minutes with 30 minutes rest between exposures. On day 27, immediately after the first Ova aerosol exposure, the mice were given an adoptive transfer of 5 x 10^6 eosinophils i.v. from one of three sources; (a) the blood of IL-$5^{-/-}$ mice that were infected with VV-HA-IL-5, (b) the allergic lungs of IL-$5^{+/+}$ mice or (c) the allergic lungs of IL-$5^{-/-}$ mice that were infected with VV-HA-IL-5 (see section V.2.1.6.1.1). Control mice received 100 µl of control vehicle (PBS) only. In other experiments some mice were given an i.v. injection of 2.4 nmol/kg eotaxin (see section V.2.2 for production and purification) or 100 µl of control vehicle [10 mM PBS/0.1% BSA (pH 7.4)], instead of an i.v. adoptive transfer of eosinophils. Blood samples were taken via the tail vein immediately prior to, and at 10 minutes (donor

eosinophil recipients) or at 30 minutes (eotaxin recipients) after injection. Eosinophils/ml of blood in these samples were determined using Discombe's method (see section II.2.2). On day 29, some mice were analysed for airways hyperreactivity (see section III.2.11) and the levels of leukocytes in the BALF quantified (see section III.2.9). Other mice, that had not been tested for airways hyperreactivity or undergone BAL, were sacrificed by cervical dislocation and their lungs processed for histological examination (see section III.2.12). The lungs of an additional group of non-sensitised IL-$5^{-/-}$ mice (given 1 mg of Alhydrogel in 0.2 mls of sterile 0.9% saline on days 0 and 12), that were exposed an aerosol of saline instead of Ova on days 24, 26, 27 and 28, also underwent histological examination and were used for comparative purposes.

V.2.1.6.1 Production of donor eosinophils from the lungs and the blood.

V.2.1.6.1.1 *Production of donor eosinophils from the lungs.*

IL-$5^{+/+}$ and IL-$5^{-/-}$ mice (male, 8-10 weeks of age) were sensitised to Ova by i.p. injection on days 0 and 12 (see section V.2.1.1). On day 23, the IL-$5^{-/-}$ mice received an i.n. inoculum of 1 x 10^7 pfu of VV-HA-IL-5. On days 24, 26, 28 and 30, the IL-$5^{+/+}$ and IL-$5^{-/-}$ mice were exposed to an aerosol of Ova (10 mg/ml) in 0.9% saline for 30 minutes, 3 times with 30 minutes rest between exposures. Mice were sacrificed on day 31 and the cells in the airways collected by BAL (see section III.2.9). The BALF cells were sequentially incubated with rat anti-mouse CD45R (B220, Pharmingen, San Diego, CA., USA.), rat anti-mouse CD2 mAb (LFA-2, Pharmingen, San Diego, CA., USA.) and goat anti-rat IgG, conjugated to MACS colloidal super-paramagnetic microbeads (Miltenyi Biotec Inc, Sunnyvale, CA., USA.). The eosinophils were separated from the CD45R-positive and CD2-positive cells by negative selection using the MiniMACS system (Miltenyi Biotec Inc., Sunnyvale, CA., USA.). The purity of the enriched population of eosinophils was > 90% as determined by differential staining with Giemsa-May-Grunwald, with neutrophils being the major contaminating cell type. Cell viability was > 98% as assessed by trypan blue exclusion.

V.2.1.6.1.2 *Production of donor eosinophils from blood.*

IL-$5^{-/-}$ mice were inoculated i.v. with 0.5 x 10^7 pfu of VV-HA-IL-5. Eight days later the mice were anaesthetised with ketamine (60 mg/kg) and rompun (8 mg/kg) by i.p. injection and their blood collected by cardiac puncture. After red cell lysis, eosinophils were purified from the blood using the MiniMACS system (see section V.2.1.6.1.1). Eosinophil purity was > 90% (see section IV.2.1.3), with neutrophils being the major contaminating cell type. Eosinophil viability was > 98% as assessed by trypan blue exclusion.

V.2.2 The production of recombinant murine eotaxin using the baculovirus expression system.

See figure V.1 for a schematic representation of the cloning of the eotaxin producing baculovirus (BV-eotaxin).

V.2.2.1 *Cloning of murine eotaxin cDNA into the baculovirus transfer vector pBacPAK8.*

Murine eotaxin cDNA (21-328 bp of full length murine eotaxin which encodes functional eotaxin) was a gift from Professor Mark Rothenberg (Childrens Hospital Medical Centre, Cincinnati, OH., USA.) and was supplied cloned in the PCRII plasmid (Invitrogen, Carlsbad, CA., USA.), with added 5' *Hin*dIII and 3' *Eco*R1 restriction enzyme sites. This plasmid was called MR#310. pBacPAK8 (1 μg) (CLONTECH Laboratories, Inc. Palo Alto, CA., USA.) was digested with *Sma* I (Boehringer Mannheim, Castle Hill, NSW., Australia) and the 5' blunt end termini were dephosphorylated using calf intestinal alkaline phosphatase (CIAP) (Boehringer Mannheim, Castle Hill, NSW., Australia) according to the manufacturer's instructions to prevent self-ligation. The CIAP was removed by treatment with proteinase K (Boehringer Mannheim, Castle Hill, NSW., Australia) according to methods described by Sambrook *et al.*, (1989). The plasmid DNA was then phenol/chloroform extracted, ethanol/acetate precipitated (Sambrook *et al.*, 1989) and resuspended in TE buffer.

MR#310 was electro-transformed into competent DH5α *E. coli* cells. Following amplification in Luria-broth (LB) supplemented with 50 μg/ml ampicillin, the transformed cells were plated onto 2% bactoagar/LB plates containing 100 μg/ml ampicillin. Five individual colonies were further amplified in LB containing 50 μg/ml ampicillin. The MR#310 plasmid was purified from these 5 broths using Wizard mini-preps (Promega, Madison, WI., USA.) according to the manufacturer's instructions and after restriction enzyme digest with *Eco*R1 and *Hin*dIII, the plasmid DNA was analysed on a 1% agarose/TAE gel to check for transformants. Transformed cells exhibited a band of DNA of approximately 310 base pairs in length, which corresponded to the size of the eotaxin cDNA sequence. Two positive transformants were then amplified in LB containing 50 μg/ml ampicillin and the MR#310 plasmid was purified using the Wizard maxi-preps system (Promega, Madison, WI., USA.) according to the manufacturer's instructions.

Eotaxin cDNA was excised from MR#310 using *Hin*dIII (5') and *Eco*R1 (3') and the resulting 5' overhangs were end filled to form blunt end termini using the Klenow

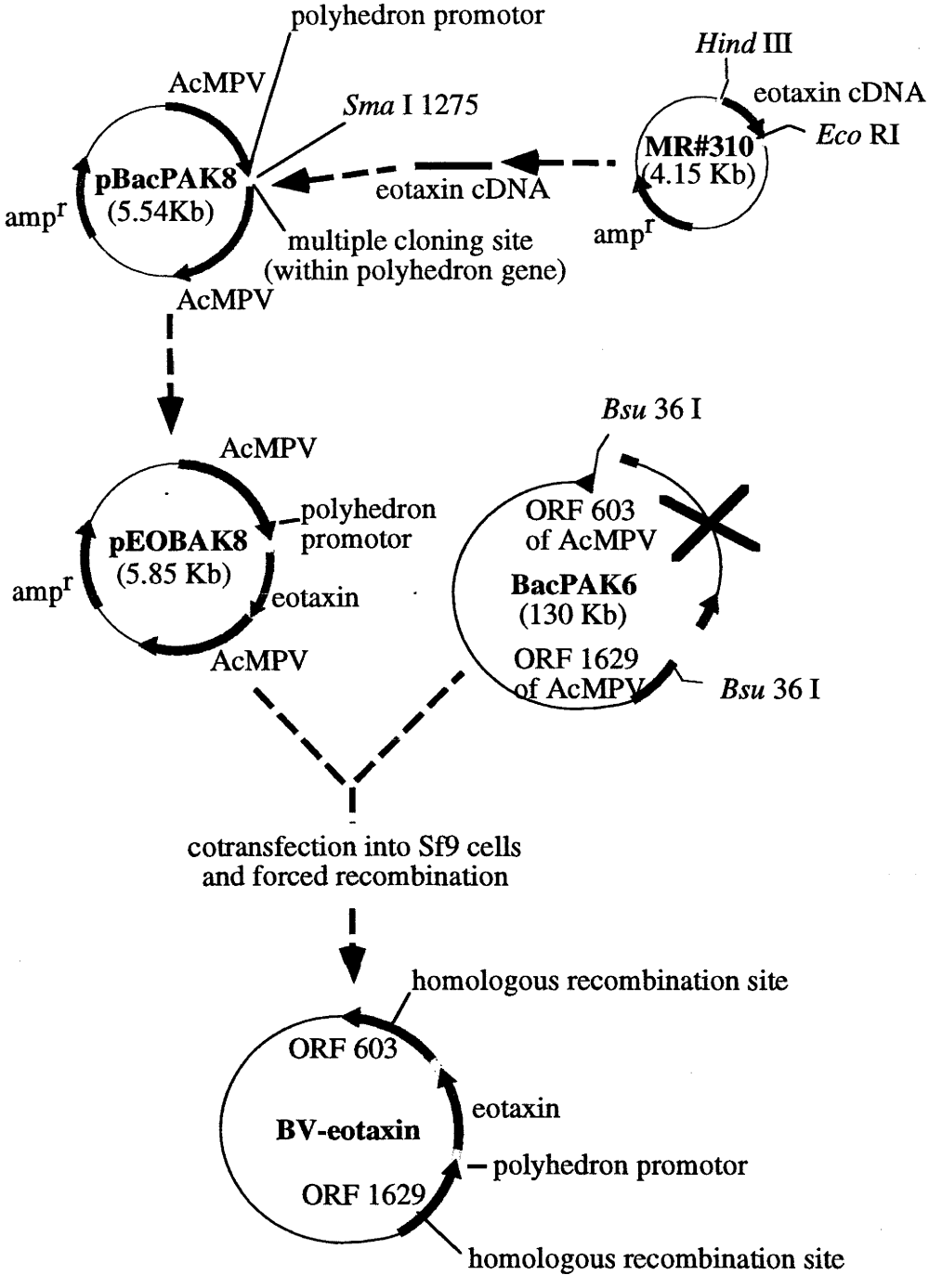


Figure V.1 ***Cloning of BV-eotaxin.***

Eotaxin cDNA was excised from MR#310 using *Hind* III and *Eco* R1 and blunt end ligated into the multiple cloning site of the baculovirus compatible vector pBacPAK8. The resulting plasmid pEOBAC8 was cotransfected with linearized (Bsu 361 digest) wildtype baculovirus (BacPAK6) into Sf9 cells. After homologous recombination of the polyhedron open reading frame (ORF 692-1629 of AcMPV) BV-eotaxin was generated.

fragment of *E. coli* polymerase I (Promega, Madison, WI., USA) according to the manufacturer's instructions. Using a molar ratio of 20:1, 108 ng of eotaxin cDNA was ligated with 100 ng of linearized pBacPAK8 by incubation with 1 Weiss unit of T4 DNA Ligase in ligase buffer (Promega, Madison, WI., USA.) according to the manufacturer's instructions. The ligation mix was electro-transformed into competent DH5α *E. coli* and the transformed cells were plated onto 2% bactoagar/LB plates containing 100 μg/ml ampicillin. Individual colonies were amplified in LB containing 50 μg/ml ampicillin and the plasmid DNA was purified using Wizard mini-preps (Promega, Madison, WI., USA.) according to the manufacturer's instructions. pBacPAK8/eotaxin positive ligations were identified by PCR using primers designed homologous to 5' and 3' regions of the ligation site (see section V.2.2.1.1).

V.2.2.1.1 Determination of positive ligation using PCR.

Plasmid DNA (5 μl) was added to a 20 μl reaction mix containing 5 pmol of each of forward primer (BAC-1) and reverse primer (BAC-2)(CLONTECH Laboratories, Inc., Palo Alto, CA., USA.), 1 unit of AmpliTaq DNA polymerase (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ., USA.) and 250 μM each of dGTP, dCTP, dATP and dTTP (Promega, Madison, WI., USA.) in AmpliTaq buffer (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ., USA.). The PCR primers, BAC 1 (5' ACCATCTCGCAAATAAATAAG 3') and BAC 2 (5' ACAACGCACAGAATCTA 3'), were designed homologous to the 5' and 3' regions (of the opposite strand) of the multiple cloning site, respectively. The PCR was performed using a FTS-1 Thermal Sequencer (Corbett Research, Sydney, Australia) using the following conditions. Denaturation at 94°C for 5 minutes, followed by 5 cycles of denaturation at 94°C for 15 seconds, annealing for at 55°C 20 seconds and extension at 72°C for 1 minute. This was followed by another 31 cycles of denaturation for 5 seconds at 94°C, annealing at 55°C for 20 seconds and extension at 72°C for 1 minute and finished with 1 cycle of denaturation at 94°C for 5 seconds, annealing at 55°C for 20 seconds and extension at 72°C for 15 minutes.

PCR products (3 μl) were analysed by polyacrylamide gel electrophoresis (PAGE, see section III.2.1.5.2.1) and were identified by size by reference to a 50-2000 base pair DNA size standard ladder (BioRad, Hercules, CA., USA.).

V.2.2.1.2 Confirmation of ligations and determination of the insert's orientation.

Plasmid DNA was purified from 8 ligation positive colonies amplified in LB under ampicillin selection using the Wizard plasmid miniprep system (Promega, Madison, WI., USA.). The orientation of the eotaxin cDNA in pBacPAK8 was determined by digestion

of each plasmid with *Bgl*II (Boehringer Mannheim, Castle Hill, NSW., Australia) and analysing the digestion products by PAGE (see section III.2.1.5.2.1). *Bgl*II digestion of a plasmid containing the eotaxin gene in the correct orientation was calculated to liberate 2 fragments of DNA of 5595 and 251 base pairs in length while an incorrect orientation was calculated to liberate 2 fragments of DNA 5665 and 181 in length. A plasmid showing correct orientation eotaxin cDNA was chosen and named pEoBAK8.

V.2.2.2 *Transfection and viral purification.*

V.2.2.2.1 Maintenance of *Spodoptera frugiperda* insect cells.

Spodoptera frugiperda (Sf9) cells were a gift from Professor Ian Young (John Curtin School of Medical Research, ANU., ACT., Australia). The cells were grown at 27°C in TNM-FH medium [Graces' insect cell culture medium (Gibco BRL, Life Technologies, Gaithersburg, MD., USA.) supplemented with 40 mM $NaHCO_3$, 0.4% yeastolate ultrafiltrate (Gibco BRL, Life Technologies, Gaithersburg, MD., USA) and 0.33% lactalbumin hydrolysate (Gibco BRL, Life Technologies, Gaithersburg, MD., USA.)] supplemented with 10% heat inactivated FCS. The cells were grown either as monolayers in plastic tissue culture flasks (Gibco BRL, Life Technologies, Gaithersburg, MD., USA.) or as spinner cultures in BELCO spinner flasks (BELCO Biotechnology, NJ., USA.). Monolayers of cells were passaged upon confluence by removing the media and gently washing adherent cells off the bottom of the tissue culture flask with fresh medium. Cells were diluted 1 in 5 in fresh media and reseeded into new culture flasks. Spinner cultures of cells were seeded at a density of 5×10^5 cells/ml into BELCO spinner flasks and were grown at 27°C on a magnetic stirrer plate set at 75 rpm. Cells in suspension cultures were passaged when their density reached 2×10^6 of cells/ml.

V.2.2.2.2 Transfection of *Spodoptera frugiperda* cells with pEoBAK8 and BacPAK6.

Two 25 cm^2 tissue culture flasks were seeded with 1.5×10^6 exponentially growing Sf9 cells in 5 mls of TNM-FH/10% FCS and were incubated overnight at 27°C. The cells were then gently washed twice (1 x 20 seconds and 1 x 15 minutes) with fresh TNM-FH, followed by the addition of 1.5 mls of fresh TNM-FH. One hundred microlitres of transfection solution [5 µl of BacPAK6 viral DNA (*Bsu*36 I digest (CLONTECH Laboratories, Inc. Palo Alto, CA., USA.), 500 ng of pEoBAK8 (see section V.2.2.1) and 50 µg Lipofectin (GIBCO BRL, Life Technologies, Gaithersburg, MD., USA.)], that had been previously incubated at room temperature in a polystyrene tube for 15 minutes, was added drop wise to one flask of cells. To the other flask of cells, only plasmid DNA was added and served as a control. After 5 hours incubation at 27°C, 1.5 mls of TNM-FH/10% FCS was added to the cells. The flasks were then incubated for a

further 3 days at 27°C. The media was collected and the cellular debris pelleted by centrifugation (400 x g, for 10 minutes at room temperature). The supernatants, containing virus, were stored at 4°C in the dark.

V.2.2.3. *Viral purification.*

V.2.2.3.1 Isolation and amplification of individual viruses by plaque assay.

Individual viruses were cloned from culture supernatants (see section V.2.2.2.2) as follows. Tissue culture dishes (35 mm, Gibco BRL, Life Technologies, Gaithersburg, MD., USA.) were seeded with 1.5 x 10^6 exponentially growing Sf9 cells in 3 mls TNM-FH/10% FCS and were incubated at 27°C for 5 hours. The media was removed and the cells were infected with logarithmic dilutions of virus containing culture supernatants in 1 ml of TNM-FH for 1 hour at room temperature on a rocking platform. The media was then removed and the cells were overlayed with 4 mls of agarose solution [0.5% SeaKem agarose (FMC BioProducts, Rockland, ME., USA.) in TNM-FH/10% FCS at 37°C]. The cells were incubated for 6 days at 27°C and the viral plaques were visualised under low power light microscopy (10 x magnification) with incident light. From a plate with well-separated plaques, individual viral plaques were harvested by cutting out an agar plug directly above the viral plaque using a sterile pasteur pipette. Individual agar plugs were placed in 1 ml TNM-FH and the virus allowed to diffuse into the medium over 24 hours at 4°C in the dark.

Viruses from each of the 8 individual agar plugs were amplified as follows. Viral solution (from agar plug in 1 ml of TNM-FH) was diluted 1 in 2 in TNM-FH and was added to a 25 cm^2 tissue culture flask of media-drained Sf9 cells, that was seeded the previous day with 1 x 10^6 exponentially growing cells. The virus was left to infect the cells for 1 hour at room temperature on a rocking platform. TNM-FH/10% FCS (4 mls) was then added to the tissue culture flask and the flask incubated for a further 3 days at 27°C. The virus was then harvested (see section V.2.2.2.2) and was stored at 4°C in the dark.

V.2.2.3.2 Checking for presence of eotaxin gene in recombinant baculoviruses.

Each of the 8 individual baculoviral stocks (section V.2.2.3.1) was checked for the presence of the eotaxin gene using PCR. Samples were prepared for PCR by centrifuging 50 µl of each viral stock at 1000 x g for 10 minutes at room temperature to remove cellular debris. The supernatants were then boiled for 10 minutes and debris pelleted by centrifugation (1000 x g for 5 minutes). The DNA in the supernatant was

precipitated using ethanol/acetate (Sambrook *et al.*, 1989) and was resuspended in 30 µl of TE buffer.

Viral DNA (5 µl) was added to a 20 µl PCR reaction mix containing 5 pmol each of forward primer (EOBACF1) and reverse primer (EOBACR1), 1 unit of AmpliTaq DNA polymerase (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ., USA.) and 250 µM each of dGTP, dCTP, dATP and dTTP (Promega) in AmpliTaq buffer (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ., USA.). To control for contamination, a water control was also run and consisted of 5 µl of PCR-grade water added to 20 µl of reaction mix. The PCR primers, EOBACF1 (5' TAGTTGCTGATATC ATGGAG AT 3') and EOBACR1 (5' ACAACGCACAGAATCTAGCG 3') were designed homologous to 5' and 3'(of the opposite strand) regions respectively of the eotaxin cDNA in pEoBAK8. The primers were designed to amplify a 564 bp section of cDNA and were synthesised at the Biomolecular Resource Facility of the John Curtin School of Medical Research School (Canberra, Australia).

The PCR was performed using a FTS-1 Thermal Sequencer (Corbett Research, Sydney, Australia) under the following conditions. Denaturation at 94°C for 3 minutes followed by 39 cycles of denaturation at 94°C for 3 seconds, annealing at 55°C for 3 seconds and extension at 72°C for 2 minute. The PCR was concluded by 1 cycle of denaturation at 94°C for 3 seconds, annealing at 55°C for 3 seconds and extension at 72°C for 9 minutes. The PCR products (3 µl) were analysed using PAGE (see section III.2.1.6.2.1). All virus clones were positive for the presence of the eotaxin gene.

V.2.2.3.3 Virus selection by analysis of eotaxin production using Western dot blot.

Each viral stock was titred in duplicate by plaque assay (see section IV.2.1.3). Flasks (25 cm^2) of SF9 cells that had been seeded the previous day with 1 x 10^6 cells, were infected with virus at a M.O.I. of 2 at room temperature for 1 hour on a rocking platform. An additional 4 mls of TNM-FH/10% FCS was then added and the culture flasks were incubated at 27°C for 3 days. The media was then harvested and centrifuged (1000 x g for 5 minutes at 4°C) to pellet the cellular debris. Supernatants containing virus were stored at 4°C in the dark.

The level of eotaxin expression by each virus clone was analysed by Western dot blot (see section III.2.13) with one adaptation; Polyclonal rabbit anti-mouse eotaxin (0.2 µg/ml) was substituted for polyclonal rabbit anti-mouse MBP as the primary antibody. Each virus was graded on its ability to express eotaxin according to the intensity of the dot blot reaction. The virus with the highest eotaxin production was chosen and denoted BV-eotaxin.

V.2.2.4 *Large scale viral stock production.*

Suspension cultures of exponentially growing Sf9 cells (500 mls of 5 x 10^5 cells/ml in TNM-FH/10% FCS, in a Belco spinner flask) were inoculated with BV-eotaxin at a M.O.I. of 0.2. The cultures were incubated at 27°C until 80% cells were dead (approximately 6-7 days) as assessed by trypan blue exclusion. Culture media (viral stock) were then harvested and the cellular debris pelleted by centrifugation (1000 x g for 5 minutes, 4°C). Viral stocks (supernatant) were stored at 4°C in the absence of light and the viral titre determined by plaque assay (V.2.2.3.1). The viral titres were usually approximately 10^8 pfu/ml.

V.2.2.5 *Production of recombinant murine eotaxin.*

Recombinant murine eotaxin was produced in suspension cultures of Sf9 cells. Briefly, spinner cultures of exponentially growing SF9 cells (2 x 10^6 cells/ml in TNM-FH/10% FCS) were infected with BV-eotaxin at a M.O.I of 2. After 3 days of incubation at 27°C, the cultures were centrifuged (1000 x g for 5 minutes) to remove cellular debris and the supernatant collected and stored at 4°C.

V.2.2.6 *Purification of recombinant murine eotaxin from BV-eotaxin culture supernatants.*

Recombinant murine eotaxin was purified from BV-eotaxin conditioned culture medium (see section V.2.2.5) to > 90% homogeneity using methods described by Kitaura *et al.*, (1996). Briefly, culture supernatants were dialysed against 3 changes of 10 mM sodium phosphate/150 mM NaCl (pH 7.4) over 24 hours. The dialysed culture supernatant (25 mls) was then loaded at a rate of 1 ml/minute onto a 1 ml HiTrap Heparin column (Pharmacia Biotech, Uppsala, Sweden) that was pre-equilibrated with 10 mM sodium phosphate buffer/150 mM NaCl (pH 7.4). After washing the column with 10 volumes of 10 mM sodium phosphate buffer/400 mM NaCl (pH 7.4), the bound protein was eluted with 10 volumes of 10 mM sodium phosphate buffer/600 mM NaCl (pH 7.4). The Elutant was diluted with 1 volume of 10 mM sodium phosphate buffer (pH 7.4) and was loaded at a rate of 1 ml/minute onto a 1 ml HiTrap SP ion exchange column (Pharmacia Biotech, Uppsala, Sweden) that was pre-equilibrated with 10 mM sodium phosphate/300 mM NaCl (pH 7.4). After washing the column with 10 volumes of 10 mM sodium phosphate/300 mM NaCl (pH 7.4), the bound protein was eluted at a rate of 1 ml/minute with a 10 ml NaCl gradient (300 mM to 1 M) in 10 mM sodium phosphate buffer. Fractions (1 ml) were collected and analysed for eotaxin content by dot blot (see section V.2.2.3.3). The fractions that contained eotaxin were pooled, dialysed against 3 changes of 10 mM sodium phosphate/150 mM NaCl (pH 7.4), over 24 hours and filter sterilised

through a 0.22 μm filter. An aliquot of purified eotaxin was stored at 4°C and the remainder was snap frozen in liquid N_2 and stored at -70°C.

Eotaxin was quantified by the BCA protein assay kit (Pierce, Rockford, Il., USA.) using BSA as the protein standard. Eotaxin homogeneity was checked by analysing samples on a 3.75% stacking/18% separating reducing polyacrylamide gel (Sambrook *et al.,* 1989). Samples were diluted 1:1 with 2 x sample loading buffer [10 mM Tris HCl (pH 8.0); 20 mM EDTA; 0.2% N-lauryloarcosine; 1.5% (w/v) Ficoll 400 (Pharmacia Biotech, Uppsala, Sweden); 0.05% (w/v) bromophenol blue; 0.05% xylene cyanol] and were denatured at 65°C for 3 minutes immediately prior to loading on the gel. The gels were run using a Mini-Protean II cell (BioRad, Hercules, CA., USA.) at 200 V for 35 minutes. The eotaxin that was produced by the baculovirus expression system was approximately equipotent as a commercially available *E. coli* -produced eotaxin when tested using the i.v. bioassay system (see section II.2.2).

V.3 RESULTS.

V.3.1 The role of IL-5 in eosinophil trafficking during cutaneous late-phase reaction in mice.

V.3.1.1 *The accumulation of eosinophils during cutaneous late-phase reaction is significantly reduced in the absence of IL-5.*

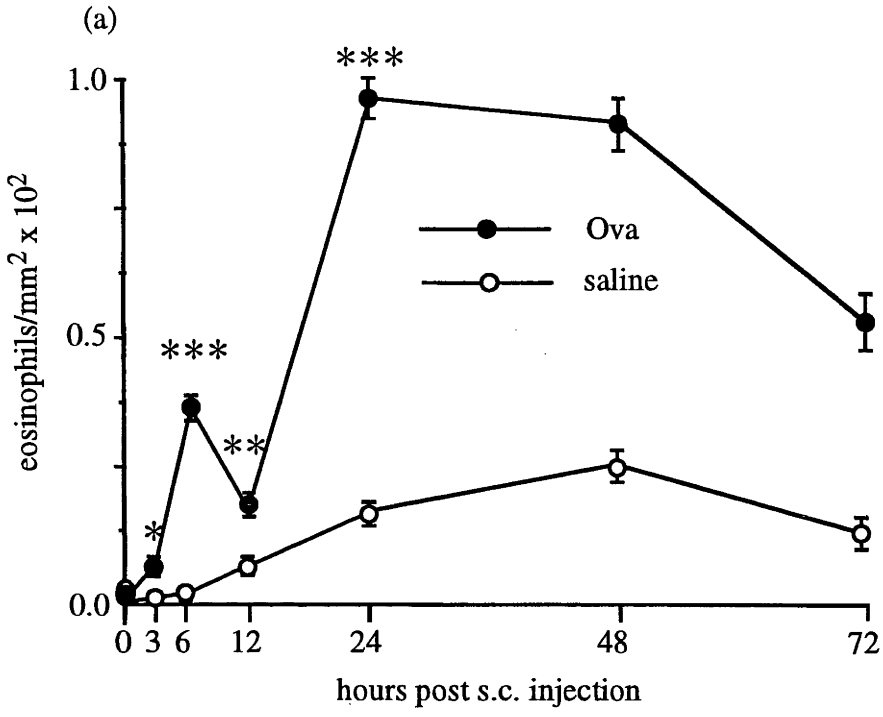
Similar to previous observations (Iwamoto *et al.*, 1992), the recruitment of eosinophils at sites of Ova-induced CLPR in mice was biphasic (figure V.2a). The first phase of eosinophil accumulation at cutaneous sites of Ova administration was transient. Eosinophils were first detected in the skin 3 hours after Ova administration, peaked at 6 hours and fell again by 12 hours. The second phase of eosinophil accumulation at sites of CLPR began at approximately 18 hours after Ova administration and peaked 6 hours later. Although peak eosinophil accumulation was observed at 24 hours, high levels of eosinophils were present at skin sites up to 48 hours post Ova administration. Furthermore, more eosinophils accumulated at sites of CLPR during the second phase than the first phase. Interestingly, the s.c. administration of saline to the non-sensitised control group also induced the accumulation of eosinophils in the skin (figure V.2a), however, the levels of this cell at these sites were significantly lower than those at the sites of CLPR. Furthermore, the accumulation of eosinophils at sites of saline administration was monophasic, unlike that seen at sites of CLPR.

The levels of eosinophils in the circulation of the sensitised IL-$5^{+/+}$ mice were significantly ($P < 0.05$) higher than those of the non-sensitised controls (figure V.2b). The levels of eosinophils in the circulation of the sensitised mice fell dramatically at 3 hours after the s.c. injection of Ova and remained at low levels up to 24 hours. This reduction correlated with the accumulation of eosinophils at sites of CLPR. In contrast, no change in the levels of eosinophils in the circulation of the non-sensitised mice were observed after they received a s.c. injection of saline (figure V.2b). This was despite the minor level of eosinophil accumulation at the s.c. injection site (figure V.2a).

The accumulation of eosinophils, at the times of peak eosinophil infiltration (6 and 24 hours, as determined in IL-$5^{+/+}$ mice), at sites of Ova-induced CLPR in the IL-$5^{-/-}$ mice was reduced in comparison to the IL-$5^{+/+}$ mice (figure V.3a). In contrast to sensitised IL-$5^{+/+}$ mice, sensitised IL-$5^{-/-}$ mice did not exhibit significantly higher levels of eosinophils in the circulation compared with the non-sensitised controls (figure V.3b). However, similar to IL-$5^{+/+}$ mice, a dramatic reduction in the levels of eosinophils in the circulation of the IL-$5^{-/-}$ mice was observed at the times of peak accumulation of eosinophils (6 and 24 hours) at sites of CLPR (figure V.3b). To determine if the

Figure V.2 ***Eosinophils trafficking during cutaneous late-phase reaction in wild type mice.***

IL-$5^{+/+}$ mice were sensitised to Ova by i.p injection on days 0 and 12 and on day 24 were given s.c. injection of 50 μg of Ova in 100 μl of saline. Non-sensitised mice were injected s.c. with saline only. (a) The accumulation of eosinophils at sites of CLPR was biphasic. Peak eosinophil accumulation was seen at 6 and 24 hours and was significantly greater than the accumulation of eosinophils at sites of saline administration in the non-sensitised controls. Results represent mean eosinophils/mm^2 ± SEM of groups of 6 animals. (b) Levels of eosinophils in the blood of mice during CLPR. Sensitised mice had significantly higher levels of eosinophils in the blood compared with the non-sensitised controls at the time of s.c. injection. Eosinophil levels in the blood of the sensitised mice fell 3 hours after s.c. Ova administration and remained low for the duration of the experiment (24 hours). In contrast, the eosinophil levels in the blood of non-sensitised control after the s.c. administration of saline were not significantly reduced. Results represent mean eosinophils/ml of blood ± SEM of groups of 6 animals. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $p < 0.05$. (a) $*P < 0.05$ when compared with saline controls at 3 hours, $**P < 0.05$ when compared to eosinophil level at 6 hours and $***P < 0.001$ when compared with saline controls at the same time point. (b) $*P < 0.05$ when compared with the saline controls at the same time point and $**P < 0.001$ when compared with the saline controls at 6 and 24 hours.


(a)
1.0
0.5
0.0
eosinophils/mm^2 x 10^2
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*
Ova
saline
0 3 6 12 24 48 72
hours post s.c. injection

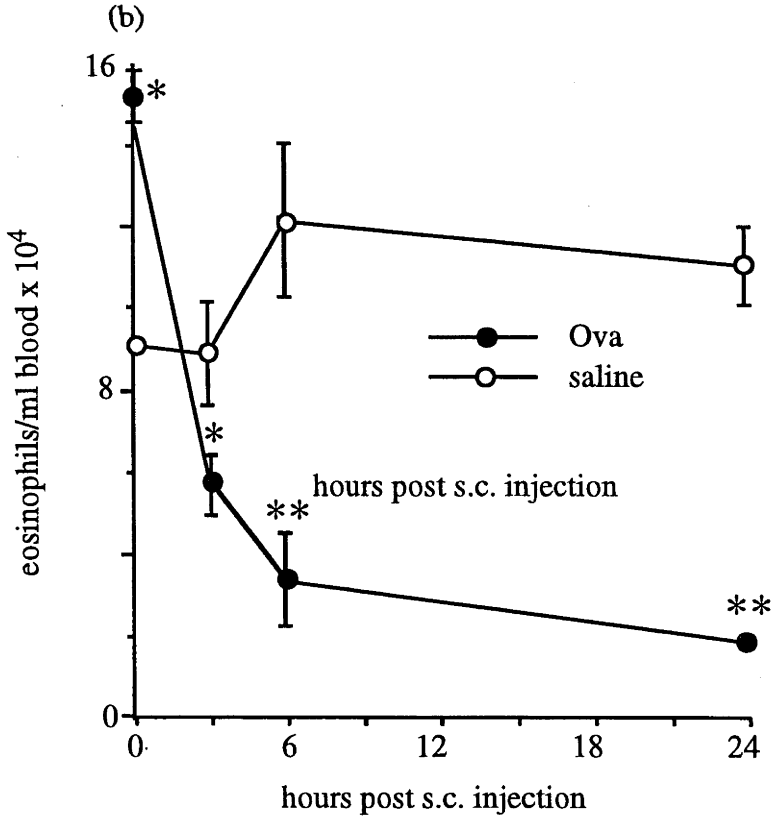
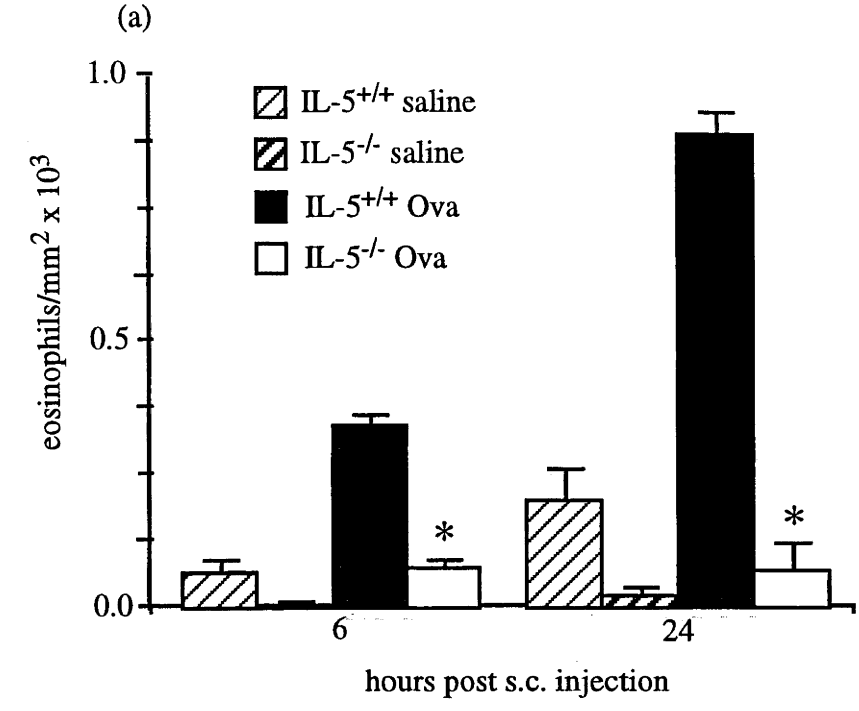
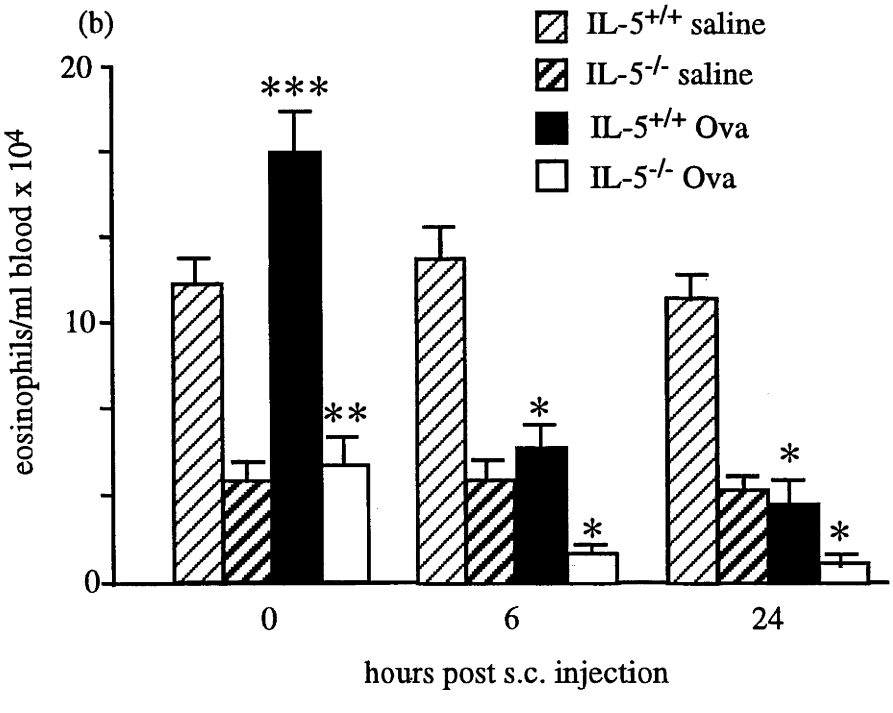

(b)
16
8
0
eosinophils/ml blood x 10^4
*
*
**
**
Ova
saline
hours post s.c. injection
0 6 12 18 24
hours post s.c. injection

Figure V.3 ***The role of IL-5 in eosinophil trafficking during cutaneous late-phase reaction in mice.***

IL-$5^{+/+}$ mice and IL-$5^{-/-}$ mice were sensitised to Ova by i.p injection on day 0 and 12 and on day 24 were challenged with 50 μg of Ova by s.c. injection in 100 μl saline. Non-sensitised mice received s.c. saline only. (a) The accumulation of eosinophils at sites of CLPR at 6 and 24 hours [peak infiltration times in wild type mice (figures IV.7 and V.2)] was significantly reduced in the IL-$5^{-/-}$ mice when compared to IL-$5^{+/+}$ mice. Saline-induced accumulation of eosinophils in the skin of the non-sensitised control animals was also reduced in the IL-$5^{-/-}$ mice when compared with the IL-$5^{+/+}$ mice. Results represent mean eosinophils/mm^2 ± SEM for groups of 6 animals. (b) Circulating eosinophil levels in IL-$5^{-/-}$ mice during CLPR. Resting levels of eosinophil in the blood of sensitised and non-sensitised mice were significantly lower than those in the IL-$5^{+/+}$ mice. Circulating eosinophils levels in the sensitised IL-$5^{+/+}$ and IL-$5^{-/-}$ mice at 6 and 24 hours post s.c. administration of Ova were significantly lower that the levels in these mice prior to Ova administration. In contrast, the levels of eosinophils in the blood of the non-sensitised controls were unaffected following s.c. saline administration. Results represent mean eosinophils/ml blood ± SEM of groups of 5 animals each. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) $*P < 0.05$ when compared with Ova challenged IL-$5^{+/+}$ mice at the same time point. (b) $*P < 0.01$ when compared to levels at 0 hours or when compared with the non-sensitised saline controls at the same time point and $**P < 0.001$ when compared with resting levels of eosinophils in the blood of the sensitised mice. No significant difference was detected between the mean resting eosinophil levels in the blood of the non-sensitised and sensitised IL-$5^{-/-}$ mice groups.


(a)
1.0
0.5
0.0
eosinophils/mm^2 x 10^3
IL-5$^{+/+}$ saline
IL-5$^{-/-}$ saline
IL-5$^{+/+}$ Ova
IL-5$^{-/-}$ Ova
*
*
6
24
hours post s.c. injection


(b)
20
10
0
eosinophils/ml blood x 10^4
IL-5$^{+/+}$ saline
IL-5$^{-/-}$ saline
IL-5$^{+/+}$ Ova
IL-5$^{-/-}$ Ova
***
**
*
*
*
*
0
6
24
hours post s.c. injection

reductions in the accumulation of eosinophils at sites of CLPR in the IL-5$^{-/-}$ mice were due to the lower circulating eosinophil levels in these animals or a decreased homing response, the trafficking of fluorescently-labelled donor eosinophils in IL-5$^{+/+}$ and IL-5$^{-/-}$ mice was analysed. The adoptive transfer of H33342-labelled donor eosinophils was confirmed in each animal by taking a blood sample 10 minutes after i.v. administration and counting the number of fluorescent cells/ml of blood. The levels of H33342-labelled eosinophils in the blood of all groups of mice (IL-5$^{+/+}$ Ova, IL-5$^{+/+}$ saline, IL-5$^{-/-}$ Ova and IL-5$^{-/-}$ saline) were equivalent at this time (figure V.4a).

Significantly greater numbers of H33342-labelled eosinophils accumulated at sites of CLPR than at skin sites of saline administration in non-sensitised mice, at 6 and 24 hours (figure V.4b). Furthermore, the accumulation of H33342-labelled eosinophils at sites of CLPR at 6 and 24 hours, or at s.c. sites of saline administration were not affected in the absence of IL-5 (figure V.4b).

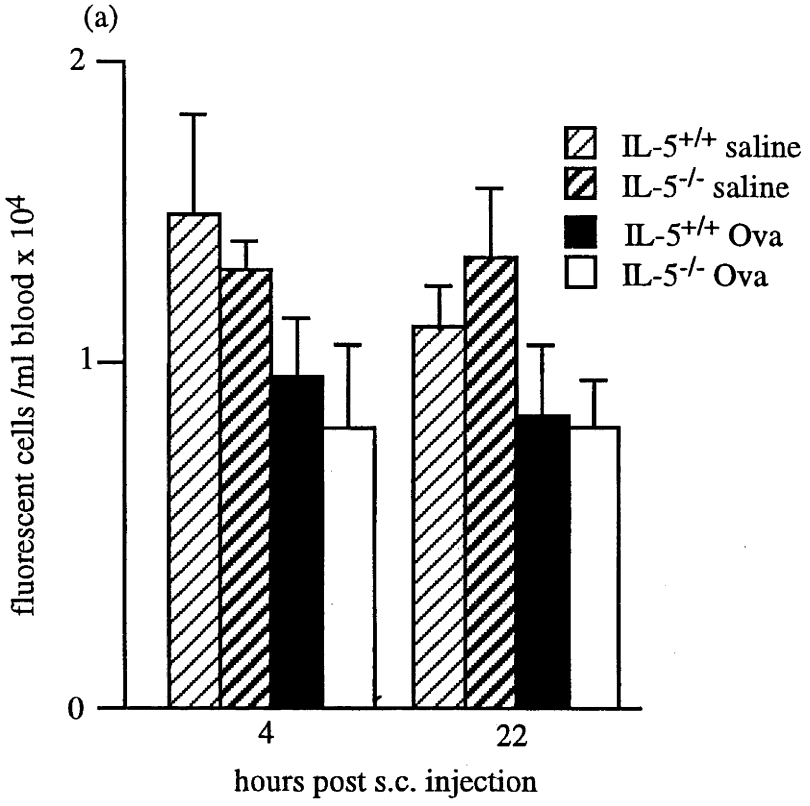
V.3.2 The role of IL-5 in eosinophil trafficking and activation during allergic airways inflammation in mice.

The single exposure of sensitised mice to an aerosol of Ova caused a time dependent increase in the level of eosinophils in the BALF of IL-5$^{+/+}$ mice, but not IL-5$^{-/-}$ mice (figure V.5a). No eosinophils were detected in the BALF of the non-sensitised control groups following their exposure to saline aerosol. A significant elevation in the level of eosinophils in the circulation of the sensitised IL-5$^{+/+}$ mice was detected between 3 and 12 hours and after 24 hours, following Ova aerosol exposure (figure V.5b). In contrast, no peripheral blood eosinophilia was detected in the sensitised IL-5$^{-/-}$ mice following Ova aerosol exposure or in the non-sensitised IL-5$^{+/+}$ and IL-5$^{-/-}$ control groups following saline aerosol exposure (figure V.5b) at any time.

The i.v. adoptive transfer of 5 x 10^6 H33342-labelled donor eosinophils to sensitised mice groups following their exposure to Ova aerosol induced the accumulation of H33342-labelled cells in the BALF 24 hours later (figure V.6a). Equivalent levels of H33342-labelled eosinophils accumulated in the lungs of IL-5$^{-/-}$ mice and IL-5$^{+/+}$ mice. In contrast, no H33342-labelled eosinophils accumulated in the BALF of the non-sensitised control groups following exposure to saline aerosol (figure V.6). The adoptive transfer of eosinophils was confirmed in each animal by taking a peripheral blood sample at 10 minutes post transfer and measuring the levels of fluorescent cell/ml of blood. The levels of H33342-labelled eosinophils in the blood of all groups of mice were equivalent at this time (figure V.6b).

Figure V.4 ***The role of IL-5 in eosinophil homing during cutaneous late-phase reaction in mice.***

IL-$5^{+/+}$ mice and IL-$5^{-/-}$ mice were sensitised to Ova by i.p injection on days 0 and 12 and on day 24 the mice were challenged with 50 μg Ova by s.c. injection in 100 μl saline. Non-sensitised mice received s.c. saline only. At 4 or 22 hours post s.c. injection, mice received an adoptive transfer of 1 x 10^6 H33342 labelled eosinophils. The mice were sacrificed 2 hours later and the accumulation of fluorescent eosinophils/mm^2 on dorsal skin membrane sections determined. (a) Fluorescent cells in the blood 10 minutes post i.v. adoptive transfer of 1 x 10^6 H33342-labelled eosinophils. Equivalent levels of fluorescent cells in the blood were present in all groups of mice at 10 minutes post adoptive transfer. Results represent mean fluorescent cells/ml blood ± SEM of groups of 5 mice each. (b) Adoptively transferred H33342-labelled eosinophils home to sites of CLPR at 6 and 24 hours and this response is not affected in IL-$5^{-/-}$ mice. Significantly greater levels of H33342-labelled eosinophil accumulation was seen at sites of CLPR than at sites of saline administration in the non-sensitised controls. Equal levels of accumulation of H33342-labelled eosinophils were seen in the IL-$5^{-/-}$ mice compared with the IL-$5^{+/+}$ mice. Results represent mean fluorescent cells/mm^2 ± SEM of groups of 5 animals. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) No significant difference between the means of any group of mice receiving an adoptive transfer of eosinophils was detected. (b) $^*P < 0.005$ when compared with saline controls at the same time point. No significant difference was detected between the means of IL-$5^{+/+}$ and IL-$5^{-/-}$ groups when compared within the same treatment.


(a)
2
1
0
fluorescent cells /ml blood x 10^4
IL-$5^{+/+}$ saline
IL-$5^{-/-}$ saline
IL-$5^{+/+}$ Ova
IL-$5^{-/-}$ Ova
4
22
hours post s.c. injection

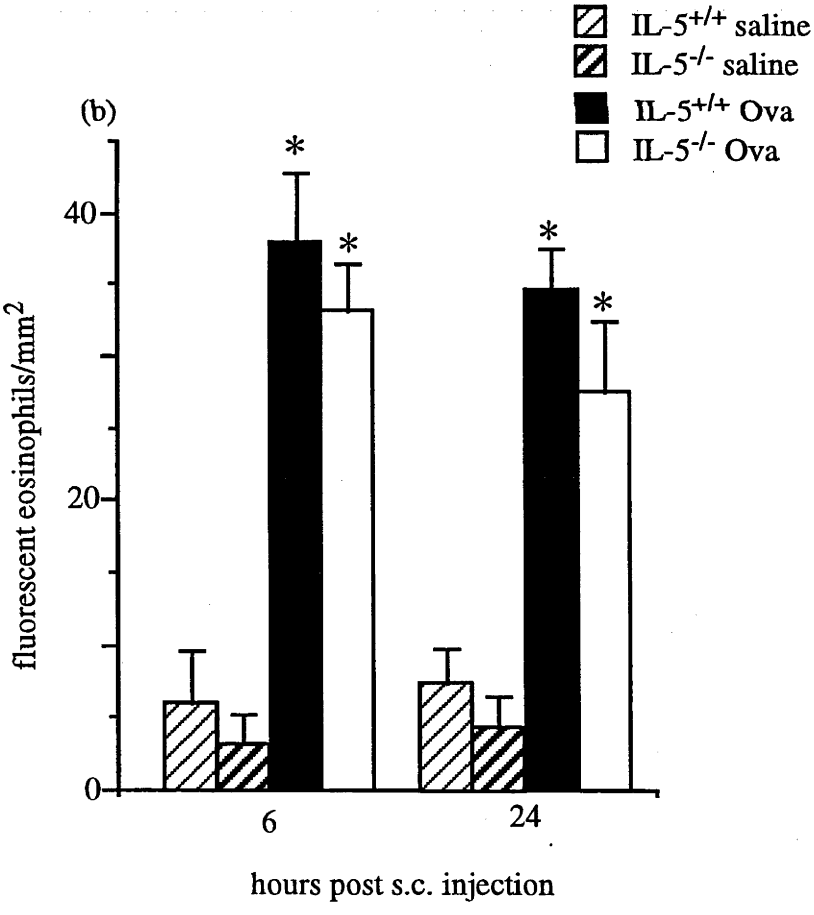
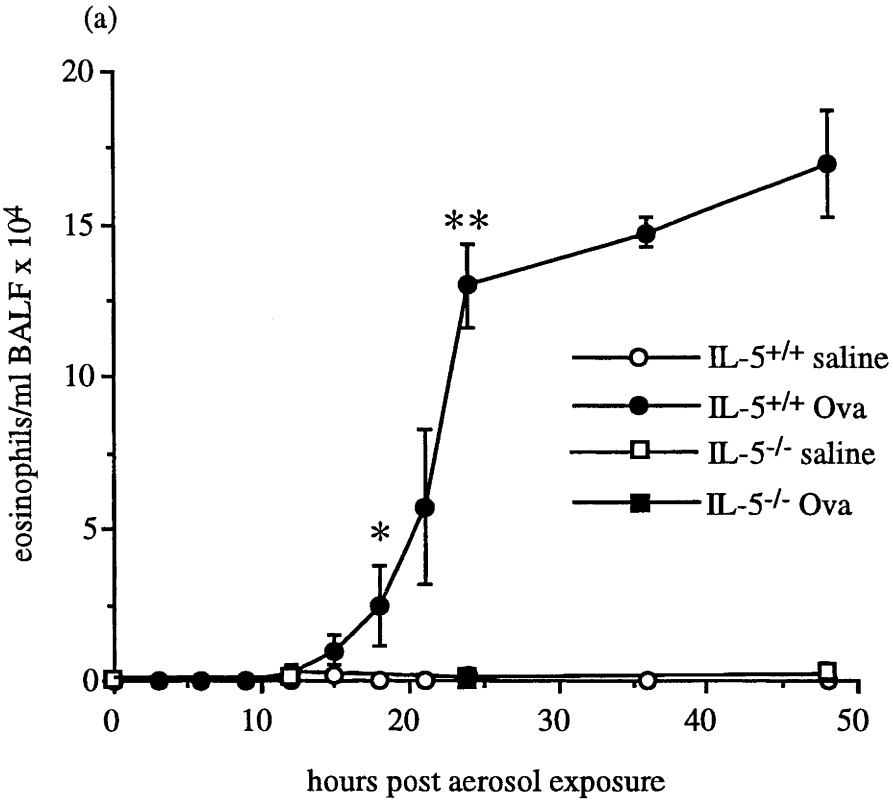

IL-$5^{+/+}$ saline
IL-$5^{-/-}$ saline
IL-$5^{+/+}$ Ova
IL-$5^{-/-}$ Ova
(b)
40
20
0
fluorescent eosinophils/mm^2
*
6
24
hours post s.c. injection

Figure V.5 ***The induction of BALF and blood eosinophilia in sensitised mice following a single exposure to aeroallergen, is attenuated in the absence of IL-5.***

IL-$5^{+/+}$ and IL-$5^{-/-}$ mice were sensitised to Ova by i.p. injection. On day 24 mice were exposed to an aerosol of Ova (10 mg/ml in saline) for a period of 1 hour. Non-sensitised mice were exposed to saline only. Circulating and BALF eosinophil levels were determined immediately prior to and every 3 hours after aerosol exposure at 24 hours and at 36 and 48 hours. (a) The exposure of sensitised mice to an Ova aerosol induced a delayed BALF eosinophilia in the IL-$5^{+/+}$ mice which (began at 18 hours and peaked by 24 hours post aerosol exposure) but not in the IL-$5^{-/-}$ mice. No significant increases in the levels of eosinophils in the BALF of the non-sensitised controls were detected following exposure to saline aerosol. Data represents mean eosinophil/ml BALF ± SEM of groups of 5 animals. (b) Antigen challenge of sensitised mice via the airways leads to the development of a rapid transient increase in circulating eosinophil levels in IL-$5^{+/+}$ mice which is abolished in the absence of IL-5. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ when compared with IL-$5^{+/+}$ saline or IL-$5^{-/-}$ Ova groups. No significant change in eosinophil levels were detected throughout the duration of the experiment in the non-sensitised controls that were exposed to saline aerosol.


(a)
20
15
10
5
0
eosinophils/ml BALF x 10^4
**
*
IL-$5^{+/+}$ saline
IL-$5^{+/+}$ Ova
IL-$5^{-/-}$ saline
IL-$5^{-/-}$ Ova
0
10
20
30
40
50
hours post aerosol exposure

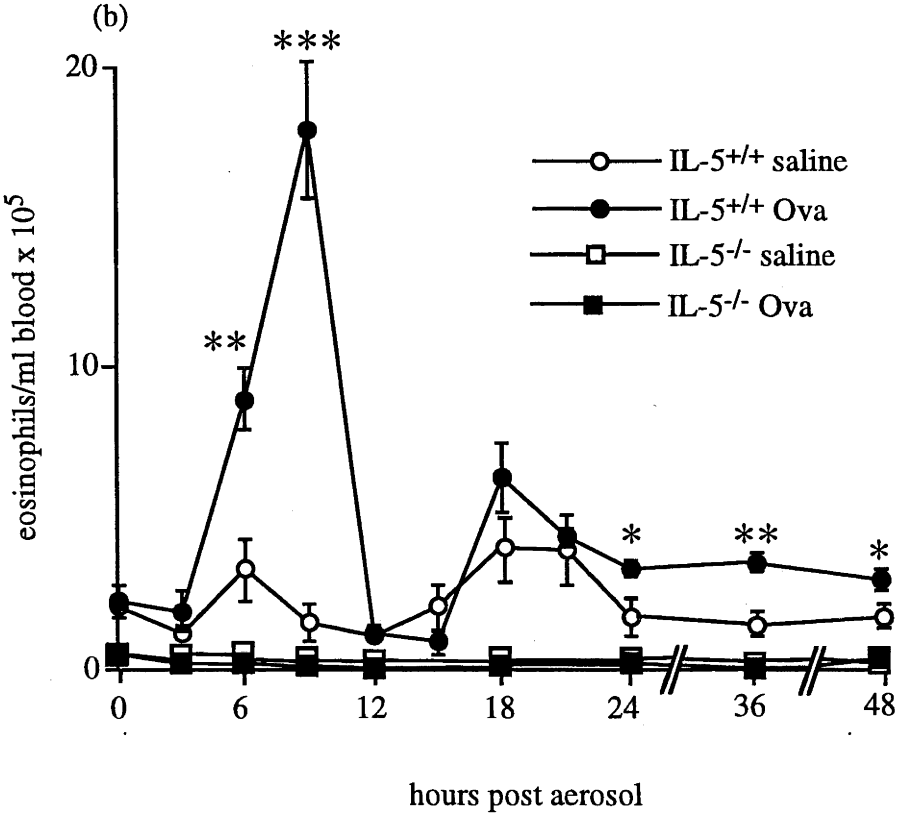
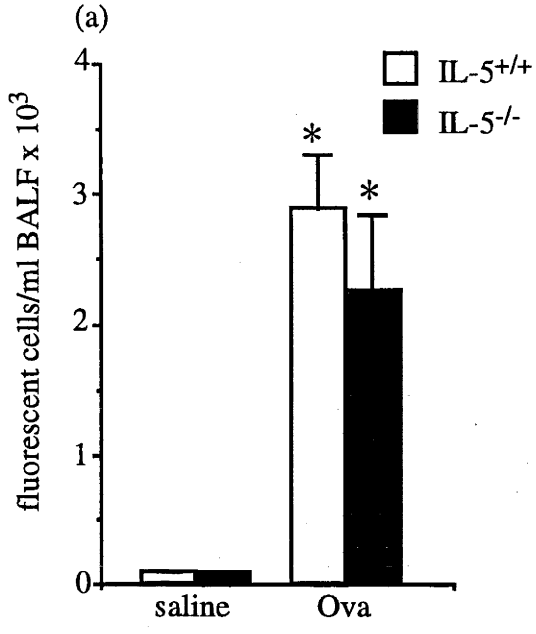
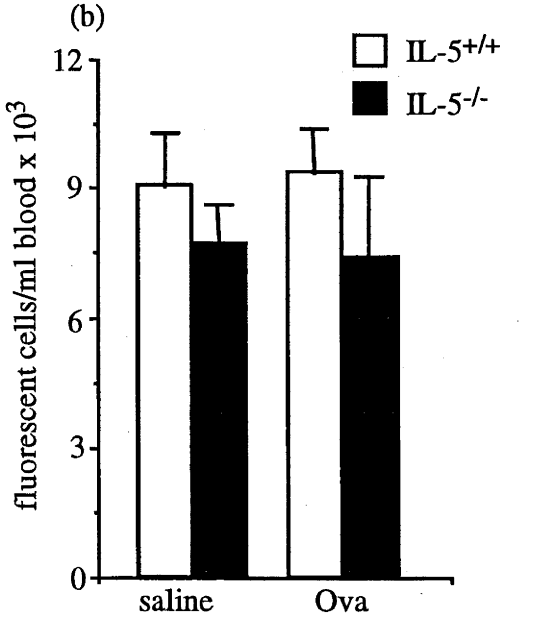

(b)
***
20
10
0
eosinophils/ml blood x 10^5
**
*
**
*
IL-$5^{+/+}$ saline
IL-$5^{+/+}$ Ova
IL-$5^{-/-}$ saline
IL-$5^{-/-}$ Ova
0
6
12
18
24
36
48
hours post aerosol

Figure V.6 ***The role of IL-5 in eosinophil homing to the allergic lungs of mice.***

IL-$5^{+/+}$ mice and IL-$5^{-/-}$ mice were sensitised to Ova by i.p injection on day 0 and 12 and on day 24 exposed to an aerosol of Ova (10 mg/ml in saline) for 1 hour. Non-sensitised mice were exposed to saline aerosol only. Six hours post aerosol exposure the mice were given an i.v. adoptive transfer of 5 x 10^6 H33342-labelled eosinophils. The levels of fluorescent cells in the BALF of mice were determined 18 hours later. (a) Fluorescent cells in BALF at 24 hours post aerosol exposure. Significantly greater levels of cells were present in the BALF of the sensitised mice when compared with the saline controls. Equivalent levels of H33342-labelled eosinophils were present in the BALF of the IL-$5^{-/-}$ mice compared with the IL-$5^{+/+}$ mice. (b) The levels of fluorescent cells in the blood at 10 minutes after transfer of H33342 labelled eosinophils. The levels of fluorescent cells in the blood of all groups of mice were equal when measured at 10 minutes post adoptive transfer. Results represent (a) mean fluorescent cells/ml BALF ± SEM of groups of 5 animals and (b) mean fluorescent cells/ml blood ± SEM of groups of 5 animals. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) $*P < 0.005$ when compared with the saline control groups at the same time point. No significant differences were detected between the means of IL-$5^{+/+}$ and IL-$5^{-/-}$ groups within same treatment. (b) No significant difference between the means of any group of mice receiving the transfer of eosinophils were detected.


(a)
IL-5+/+
IL-5-/-
fluorescent cells/ml BALF x 10^3
4
3
2
1
0
*
*
saline
Ova


(b)
IL-5+/+
IL-5-/-
fluorescent cells/ml blood x 10^3
12
9
6
3
0
saline
Ova

V.3.2.1 *Eosinophil and lymphocyte levels in BALF correlate with the degree of airways hyperreactivity in IL-5$^{+/+}$ and IL-5$^{-/-}$ mice.*

As previously demonstrated in this laboratory (Foster *et al.,* 1996), aeroallergen (Ova) exposure of sensitised IL-5$^{+/+}$ mice induced BALF eosinophilia (figure V.7a). Elevated levels of lymphocytes were also observed in the BALF of these mice (figure V.7b). The level of eosinophils and lymphocytes in the BALF correlated with the amount of aeroallergen exposures, furthermore, the BALF levels of both of these leukocytes correlated with the degree of airways hyperreactivity (figure V.7c). Following the first Ova aerosol exposure both neutrophil and macrophage levels in the BALF were elevated, however, subsequent exposures of mice to Ova aerosol failed to further increase the levels of these cells in the BALF (figure V.7b).

The induction of airways hyperreactivity occurred only in the sensitised mice and only following exposure to Ova aerosol. Non-sensitised mice that were exposed to saline aerosol did not develop airways hyperreactivity. Earlier studies in chapter 3 showed that the exposure of naive mice to an aerosol of Ova every second day for up to 10 days does not alter airways reactivity and thus, this group was not included in this study. Free MBP was exclusively detected in the BALF of the Ova sensitised mice, but only following exposure to Ova aerosol. Furthermore, the BALF levels of this cationic protein in these animals appeared to increase with subsequent exposures to aeroallergen. No Free MBP was detected in the BALF of the non-sensitised mice groups at any time.

As previously observed (Foster *et al.,* 1996), the induction of airways eosinophilia in IL-5$^{-/-}$ mice during allergic airways inflammation was significantly inhibited. In contrast to the IL-5$^{+/+}$ mice, few eosinophils were detected in the BALF of sensitised IL-5$^{-/-}$ mice when measured at 24 hours after a single exposure to Ova aerosol. However, subsequent exposures of these animals to Ova aerosol induced a slight, but significant ($P < 0.05$) increase in the levels of eosinophils in the BALF (figure V.8a). These levels were approximately 100 fold less than those observed in IL-5$^{+/+}$ mice under the same conditions (V.7a). As observed in the IL-5$^{+/+}$ mice, increases in the level of eosinophils in the BALF of the IL-5$^{-/-}$ mice also correlated with the number of Ova aerosol exposures. Free MBP was also detected in the BALF of the sensitised IL-5$^{-/-}$ mice, but only after the 3 day of exposure to Ova aerosol (table V.1).

Interestingly, although it has previously been reported to be totally abolished in IL-5$^{-/-}$ mice, airways hyperreactivity to i.v. β-methylcholine was detected in the sensitised IL-5$^{-/-}$ mice following exposure to Ova aerosol (figure V.8b), albeit at a significantly lower level than the IL-5$^{+/+}$ mice (figure V.7b) under the same conditions. A significant (when compared on Day 29 at doses > 175ng/ml β-methylcholine - $P < 0.05$.)

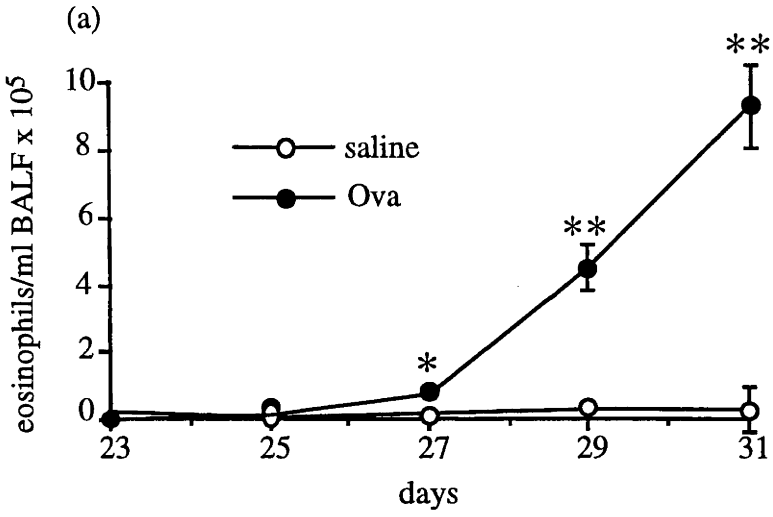
Table V.1 ***Free MBP levels in the BALF of IL-5$^{+/+}$ and IL-5$^{-/-}$ mice during allergic airways inflammation.***

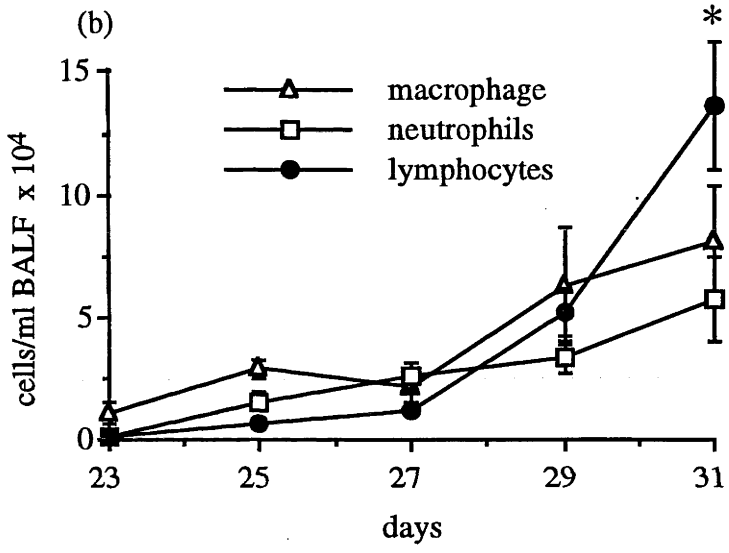
	Days of aerosol exposure				
Group	0 (day 23)	1 (day 25)	2 (day 27)	3 (day 29)	4 (day 31)
IL-5$^{+/+}$, saline	X	X	X	X	X
IL-5$^{+/+}$, Ova	X	√	√√	√√√	√√√√
IL-5$^{-/-}$, saline	X	X	X	X	X
IL-5$^{-/-}$, Ova	X	X	√	√	√√

The relative levels of free MBP in the BALF of IL-5$^{+/+}$ and IL-5$^{-/-}$ mice following aeroallergen or saline aerosol exposure were determined using immuno dot blot. Ticks indicated the intensity of the MBP staining on immuno dot blots and a cross denotes that MBP was not detected. Increasing levels of MBP were detectable in cell free BALF of sensitised IL-5$^{+/+}$ mice (IL-5$^{+/+}$, Ova) following aeroallergen (Ova) exposure. MBP levels in these mice correlated with the amount of aerosol exposure. Free MBP was also detected in the cell free BALF of the sensitised IL-5$^{-/-}$ mice, but only following 2 days of aerosol exposure. Similar to the IL-5$^{+/+}$ mice, MBP levels in the BALF of the sensitised IL-5$^{-/-}$ mice correlated with the amount of aerosol exposures. No MBP was detected in the cell-free BALF of the non-sensitised IL-5$^{+/+}$ (IL-5$^{+/+}$, saline) and IL-5$^{-/-}$ (IL-5$^{-/-}$, saline) groups prior to or post saline aerosol exposures.

Figure V.7 ***The induction of airways hyperreactivity correlates with the levels of eosinophils in the BALF following aeroallergen provocation.***

IL-5$^{+/+}$ mice were sensitised to Ova by i.p. injection on days 0 and 12. On day 24, 26, 28 and 30 the mice were exposed to an aerosol of Ova in saline. For comparative purposes another group of non-sensitised IL-5$^{+/+}$ mice were exposed to and aerosol of saline. Airways hyperreactivity to i.v. b-methylcholine and the levels of leukocytes in the BALF were determined on days 23, 25, 27 and 29 and 31. (a) Eosinophil levels in the BALF of sensitised IL-5$^{+/+}$ mice correlated with the levels of Ova aerosol exposure. No eosinophils were present in the BALF of non-sensitised controls following saline aerosol exposure. (b) Lymphocyte (but not neutrophils or macrophages) levels in the BALF of sensitised IL-5$^{+/+}$ mice correlated with the levels of Ova aerosol exposure. (c) Airways hyperreactivity was first detected in mice on day 27 and further increases in reactivity correlated with the level of Ova aerosol exposure. No differences were detected between the saline aerosol exposed group or the Ova sensitised mice prior to and 24 hours after the first exposure to Ova aerosol (data not shown). Data represents (a and b) mean cells/ml of BALF ± SEM of groups of 5 mice and (c) mean percentage airways occlusion ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) $*P < 0.01$ compared with non-sensitised saline exposed mice and $**P < 0.005$ when with levels in the same group 48 hours earlier. (b) $*P < 0.05$ when compared with the levels of lymphocytes in these mice prior to Ova aerosol exposure (day 23). (c) $*P < 0.05$ and $**P < 0.01$ compared with non-aerosol exposed sensitised mice (day 23) at the same dose of β-methylcholine.


(a)
eosinophils/ml BALF x 10^5
saline
Ova
days


(b)
cells/ml BALF x 10^4
macrophage
neutrophils
lymphocytes
days

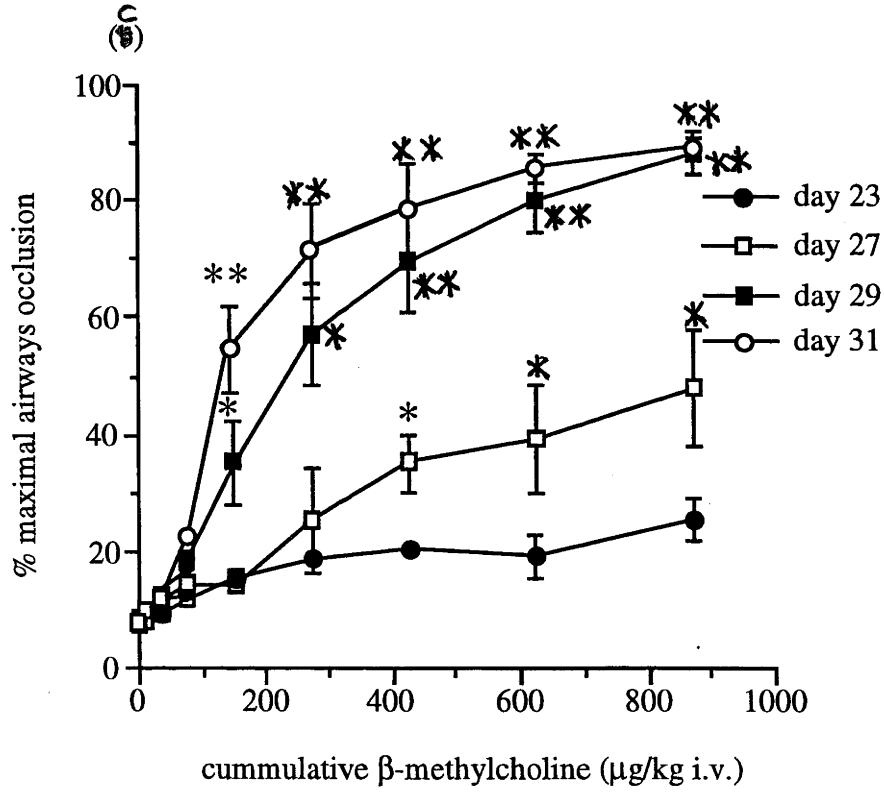
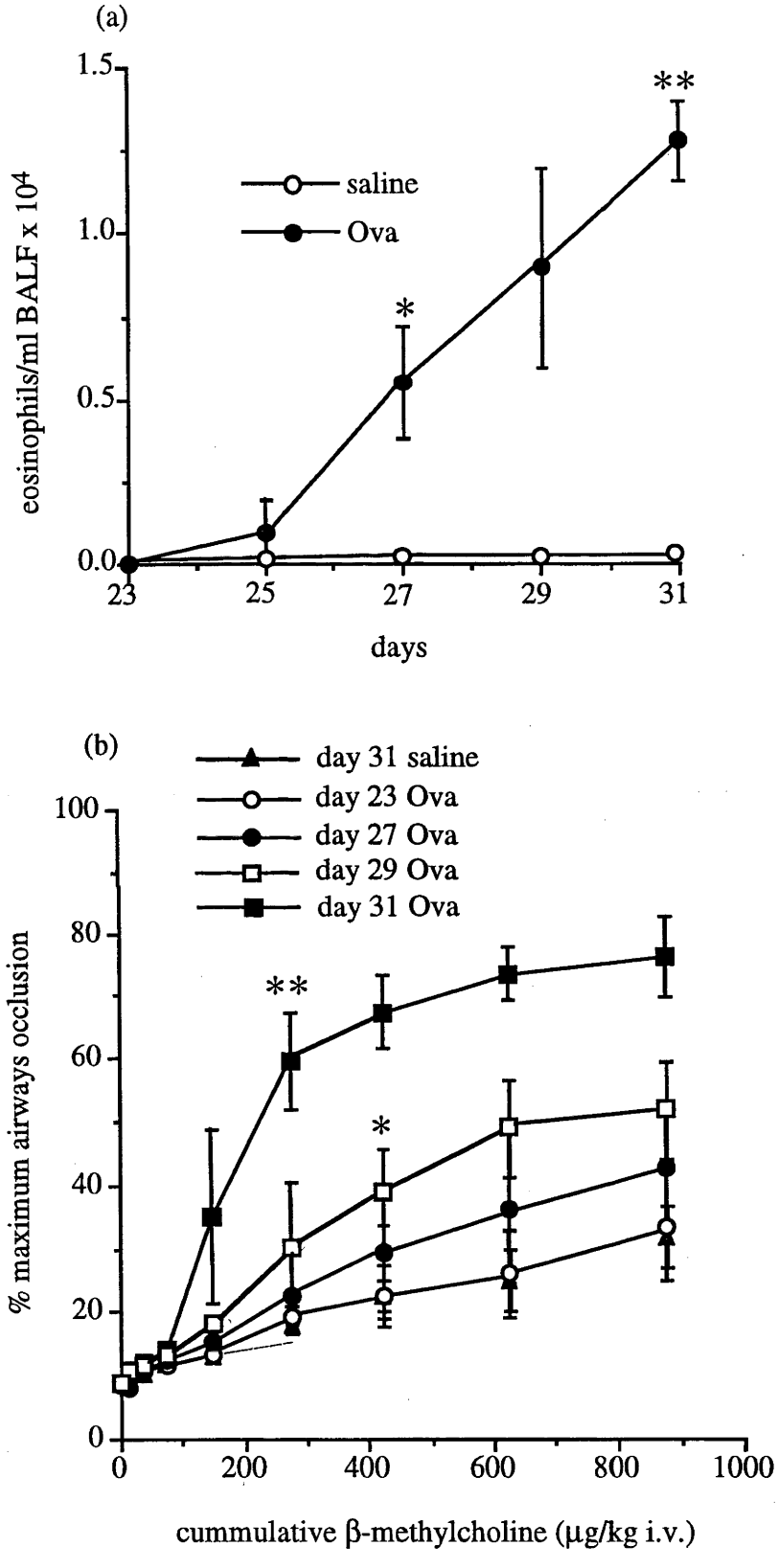

(c)
% maximal airways occlusion
day 23
day 27
day 29
day 31
cummulative β-methylcholine (μg/kg i.v.)

Figure V.8 ***BALF eosinophilia and the development of airways hyperreactivity is significantly inhibited in IL-5$^{-/-}$ mice following aeroallergen provocation and levels of BALF eosinophils correlates with the degree of airways hyperreactivity.***

IL-5$^{-/-}$ mice were sensitised to Ova by i.p. injection on day 0 and 12. On day 24, 26, 28 and 30, mice were exposed to an aerosol of Ova in saline. For comparative purposes another group of non-sensitised IL-5$^{-/-}$ mice were exposed to an aerosol of saline, instead of Ova. Mice were analysed for airways hyperreactivity to i.v. β-methylcholine and BALF eosinophil levels determined on days 23, 25, 27, 29 and 30. (a) Increases in the levels of eosinophils in the BALF correlated with the amount of exposure to Ova aerosol. Few eosinophils were present in the BALF of the non-sensitised, saline aerosol exposed mice. Data represents the mean number of eosinophil/ml BALF ± SEM of groups of 5 mice. (b) Airways hyperreactivity was first detected in sensitised the IL-5$^{-/-}$ mice on day 29 and reactivity was further increased on day 31. No differences were detected between saline aerosol exposed mice or Ova sensitised mice prior to and 24 hours after the first or second exposures to Ova aerosol (data not shown for 24 hours after the first aerosol). Data represent the mean percentage airways occlusion ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) $*P < 0.05$ compared with non-sensitised saline exposed mice and $**P < 0.05$ when compared with the sensitised mice on day 27. (b) $*P < 0.05$ and $**P < 0.01$ when compared with the non-aerosol exposed sensitised mice (day 23) at the same dose of β-methylcholine and compared with saline controls.


(a)
1.5
1.0
0.5
0.0
eosinophils/ml BALF x 10⁴
saline
Ova
*
**
23
25
27
29
31
days
(b)
day 31 saline
day 23 Ova
day 27 Ova
day 29 Ova
day 31 Ova
100
80
60
40
20
0
% maximum airways occlusion
**
*
0
200
400
600
800
1000
cummulative β-methylcholine (μg/kg i.v.)

elevation in airways reactivity of the sensitised IL-5$^{-/-}$ mice was first detected on day 29 (figure V.8b, following the third day of exposure to Ova aerosol). This was in contrast to the sensitised IL-5$^{+/+}$ mice that displayed airways hyperreactivity as earlier as day 27 (figure V.7b, 24 hours after the second exposure to Ova aerosol). It is of particular interest to note that the onset of airways hyperreactivity in the sensitised IL-5$^{-/-}$ mice occurred at the same time as free MBP was first detectable in the BALF of these animals (see table V.1).

Similar to the sensitised IL-5$^{+/+}$ mice, there was a correlation between the level of eosinophils and the presence of free MBP in the BALF, and the degree of airways hyperreactivity in the sensitised IL-5$^{-/-}$ mice on day 29. Interestingly, the levels of eosinophils in the BALF of the sensitised IL-5$^{-/-}$ mice at times when airways hyperreactivity was first detected (day 29, after 3 Ova aerosol exposures), were significantly lower than the levels of this cell in the BALF of the sensitised IL-5$^{+/+}$ mice at the onset of airways hyperreactivity (see table V.1). No airways hyperreactivity was seen in IL-5$^{+/+}$ mice 24 hours after the first exposure to Ova aerosol, even though these mice had MBP in the BALF (see table V.1) and had levels of eosinophils in the BALF (V.7a) that were equivalent to the sensitised IL-5$^{-/-}$ mice on day 31 (figure V.8a).

V.3.3 A role for IL-5 in the enhancement of eosinophil-mediated airways hyperreactivity in IL-5$^{-/-}$ mice during allergic airways inflammation.

In an attempt to further elucidate the role of eosinophils in the induction of airway hyperreactivity and to determine the contribution of IL-5 to this processes, IL-5$^{-/-}$ mice were manipulated by the i.v. administration of eotaxin or the i.v. adoptive transfer of eosinophils to amplify the eosinophilic response in the lungs in response to aeroallergen provocation. In contrast to the sensitised IL-5$^{+/+}$ mice (figure V.7a), the sensitised IL-5$^{-/-}$ mice only developed a very mild airways eosinophilia following exposure to aeroallergen (figure V.8a). The reduced accumulation of eosinophils in the BALF of sensitised IL-5$^{-/-}$ mice may reflect the lower circulating levels of eosinophils in these animals. It was thus hypothesised that by increasing the circulating eosinophil pool it would be possible to enhance the accumulation of eosinophils in BALF of IL-5$^{-/-}$ mice during allergic airways inflammation and thereby to determine the effect of the selective increase in eosinophil levels in the BALF on the development of airways hyperreactivity.

Two methods were employed to amplify the circulating eosinophil levels in the IL-5$^{-/-}$ mice during allergic airways inflammation. The first involved the i.v. administration of eotaxin (2.4 nmol/kg, see section IV.3.1) which allowed for the study of the role of eosinophils in the induction of airways hyperreactivity during allergic airways inflammation in the absence of IL-5. The second method involved supplementing the

circulating eosinophil pool by i.v. adoptive transfer of donor eosinophils. For adoptive transfer experiments, eosinophils were isolated from three sources; (a) the BALF of allergic IL-$5^{+/+}$ mice, (b) the BALF of allergic IL-$5^{-/-}$ mice inoculated with VV-HA-IL-5 (to induce a blood eosinophilia) and (c) the blood of non-sensitised IL-$5^{-/-}$ mice inoculated with VV-HA-IL-5. This provided three different populations of eosinophils that were (a) exposed to allergic mediators (including IL-5) and able to produce IL-5, (b) exposed to allergic mediators and IL-5, but unable to produce IL-5 and (c) exposed to IL-5, but unable to produce IL-5. The use of these three different populations of eosinophils allowed for the study of the effect of the activation of eosinophils by allergic mediators, the effect of IL-5 priming on eosinophils and the production of IL-5 by the eosinophils, on the development of airways hyperreactivity during allergic airways inflammation.

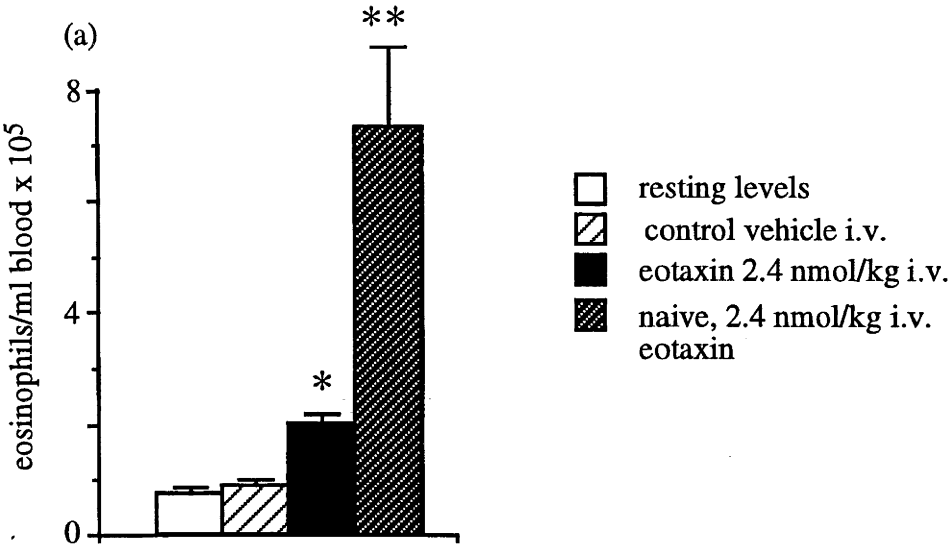
The i.v. administration of eotaxin to IL-$5^{-/-}$ mice during an allergic airways inflammatory reaction caused a significant elevation in the peripheral eosinophil levels when measured 30 minutes after administration. However, the effect of i.v. eotaxin on the circulating eosinophil pool in the allergic IL-$5^{-/-}$ mice was diminished in comparison with the effect of this chemokine on naive IL-$5^{-/-}$ mice when administered i.v. at the same concentration (figure V.9a).

The adoptive transfer of 5 x 10^6 donor eosinophils from the 3 donor sources induced significant elevations in the levels of eosinophils in the blood of the sensitised IL-$5^{-/-}$ mice when measured 10 minutes after the adoptive transfer (figure V.9b). Thus, both of these methods were effective in elevating the circulating eosinophil pool. The elevation of the circulating eosinophil pool by i.v. eotaxin or by adoptive transfer eosinophils (irrespective of their source) were equivalent in their effect on the amplification of BALF eosinophilia. Furthermore, all of these treatments selectively increased eosinophils levels in the BALF (figure V.10 a). No significant increase in the numbers of neutrophils, lymphocytes or macrophages in the BALF were observed in any of the treatment groups (figure V.10b). Interestingly, the peak circulating eosinophilia induced by i.v. eotaxin (30 minutes post i.v. administration) was significantly lower than that induce by the i.v. adoptive transfer of 5 x 10^6 eosinophils (when measured at 10 minutes post adoptive transfer).

The histological analysis of the lung sections from the sensitised IL-$5^{-/-}$ mice revealed low level of eosinophils in tissues. The accumulation of eosinophils in pulmonary tissues was enhanced in sensitised IL-$5^{-/-}$ mice that were given i.v. eotaxin or an i.v. adoptive transfer of 5 x 10^6 eosinophils 48 hours earlier (figure V.11). The eosinophils in all groups of sensitised IL-$5^{-/-}$ mice were specifically localised to the region beneath the airways smooth muscle, but not in the surrounding tissues (the parenchyma or between the airways smooth muscle and the bronchial epithelium) (figure V.12).

Figure V.9 ***The adoptive transfer of 5 x 10^6 donor eosinophils or an i.v. administration of eotaxin, significantly amplifies circulating eosinophil levels during allergic airways inflammation in IL-$5^{-/-}$ mice.***

IL-$5^{-/-}$ mice were sensitised to Ova by i.p injection on days 0 and 12 and exposed to aerosolized Ova on days 24, 26, 27 and 28. On day 27 some mice received an adoptive transfer of 5 x 10^6 donor eosinophil isolated either from the BALF of allergic IL-$5^{+/+}$ mice, the BALF of allergic IL-$5^{-/-}$ mice inoculated with VV-HA-IL-5 or the blood of IL-$5^{-/-}$ mice inoculated with VV-HA-IL-5. Mice were also injected i.v. with eotaxin (2.4 nmol/kg) or control vehicle [100 µl of 10 mM PBS/0.1% BSA (pH 7.4)] instead of an adoptive transfer of eosinophils. (b) The adoptive transfer of 5 x 10^6 donor eosinophils or (a) the i.v. administration of 2.4 nmol/kg eotaxin, significantly amplified circulating eosinophils in IL-$5^{-/-}$ during allergic pulmonary inflammation. Elevations in blood eosinophil levels induced by i.v. eotaxin (2.4 nmol/kg) in IL-$5^{-/-}$ mice during allergy were significantly lower than those induced by i.v. eotaxin, at the same concentration, in naive IL-$5^{-/-}$ mice (refer to figure IV.2b). Blood samples were analysed prior to (resting level) and (a) 10 minutes post i.v. injection of donor eosinophil recipients or control vehicle (PBS) or (b) 30 minutes post i.v. eotaxin or control vehicle (PBS/0.1% BSA). Data represents mean eosinophils/ml of blood +/- SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (b) $*P < 0.001$ compared with resting levels of control vehicle i.v. (a) $*P < 0.05$ when compared with resting levels or control vehicle i.v. and $**P < 0.002$ when compared with i.v. eotaxin (2.4 nmol/kg).


(a)
eosinophils/ml blood x 10^5
8
4
0
*
**
resting levels
control vehicle i.v.
eotaxin 2.4 nmol/kg i.v.
naive, 2.4 nmol/kg i.v. eotaxin

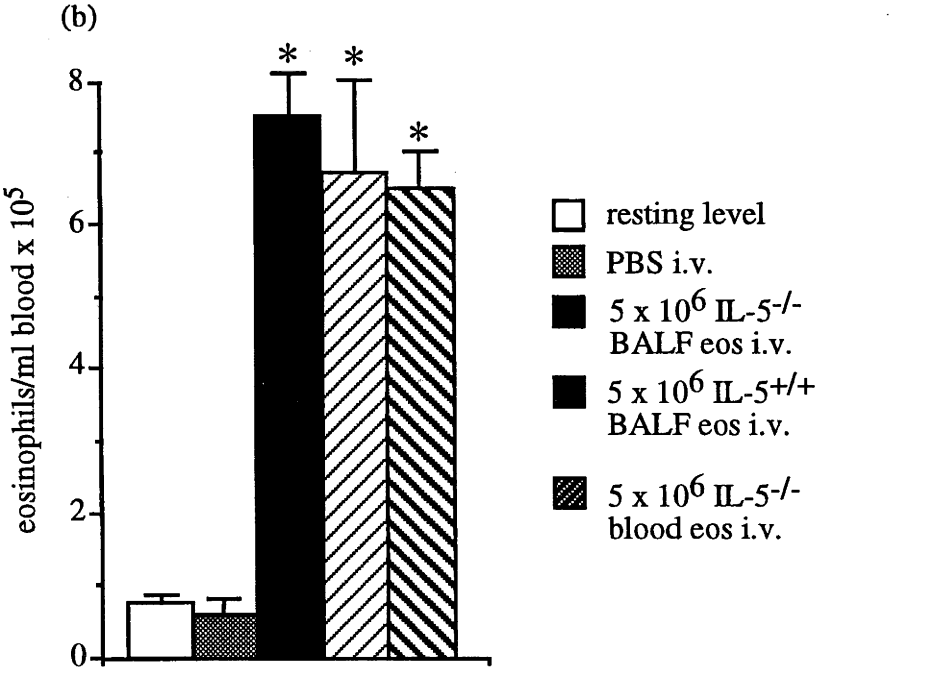
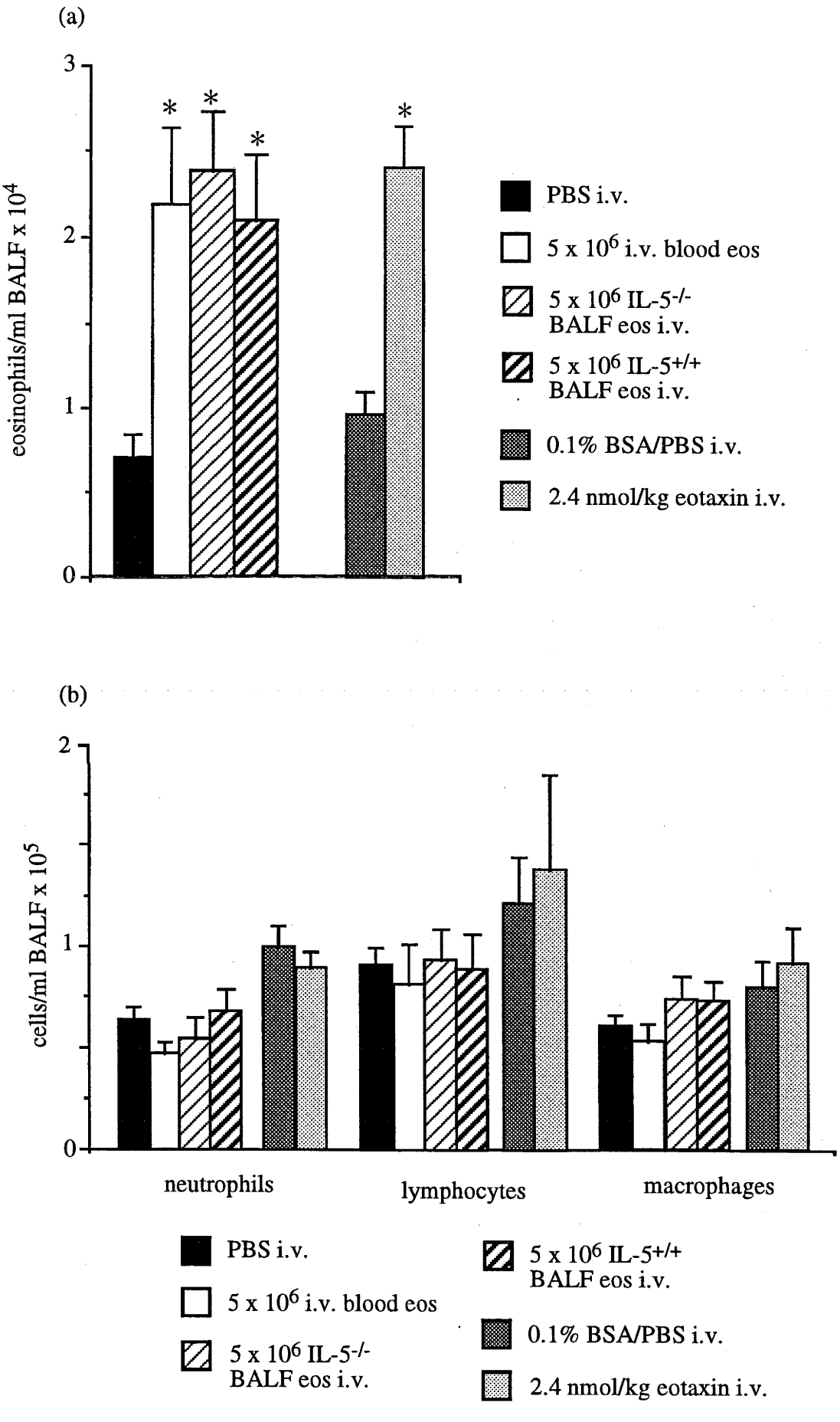

(b)
eosinophils/ml blood x 10^5
8
6
4
2
0
*
*
*
resting level
PBS i.v.
5 x 10^6 IL-$5^{-/-}$ BALF eos i.v.
5 x 10^6 IL-$5^{+/+}$ BALF eos i.v.
5 x 10^6 IL-$5^{-/-}$ blood eos i.v.

Figure V.10 ***The adoptive transfer of 5 x 10^6 donor eosinophils or the i.v. administration of eotaxin, significantly amplifies BALF eosinophil levels during allergic airways inflammation in IL-$5^{-/-}$ mice.***

The adoptive transfer of 5 x 10^6 donor eosinophils or the i.v. administration of 2.4 nmol/kg eotaxin significantly amplified (a) BALF eosinophil levels but not (b) BALF neutrophil, lymphocyte or macrophage levels in IL-$5^{-/-}$ mice during allergic airways inflammation. IL-$5^{-/-}$ mice were sensitised to Ova by i.p injection on days 0 and 12 and exposed to Ova aerosol on days 24, 26, 27 and 28. On day 27 some mice received an adoptive transfer of 5 x 10^6 donor eosinophil isolated either from the BALF of allergic IL-$5^{+/+}$ mice, the BALF of allergic IL-$5^{-/-}$ mice inoculated with VV-HA-IL-5 or the blood of IL-$5^{-/-}$ mice inoculated with VV-HA-IL-5 or control vehicle (PBS). Another group of mice was injected i.v. with eotaxin (2.4 nmol/kg) or control vehicle [10 mM PBS/0.1% BSA (pH 7.4)] instead of an adoptive transfer of eosinophils. BALF leukocyte populations were quantified on day 30 and data represents mean eosinophils/ml of BALF ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) $^*P < 0.05$ compared with control vehicle i.v. [PBS for eosinophil i.v. recipients or PBS/0.1% BSA for eotaxin i.v. injected mice]. (b) No significant differences were found between the means of groups receiving an i.v. adoptive transfer of eosinophil or an i.v. administration of eotaxin and their respective i.v. vehicle alone controls when compared within BALF leukocyte populations.


(a)
eosinophils/ml BALF x 10^4
3
2
1
0
*
*
*
*
PBS i.v.
5 x 10^6 i.v. blood eos
5 x 10^6 IL-$5^{-/-}$ BALF eos i.v.
5 x 10^6 IL-$5^{+/+}$ BALF eos i.v.
0.1% BSA/PBS i.v.
2.4 nmol/kg eotaxin i.v.
(b)
cells/ml BALF x 10^5
2
1
0
neutrophils
lymphocytes
macrophages
PBS i.v.
5 x 10^6 i.v. blood eos
5 x 10^6 IL-$5^{-/-}$ BALF eos i.v.
5 x 10^6 IL-$5^{+/+}$ BALF eos i.v.
0.1% BSA/PBS i.v.
2.4 nmol/kg eotaxin i.v.

Associated with the increase in the accumulation of eosinophils in the lungs of sensitised IL-5$^{-/-}$ mice by i.v. eotaxin or an adoptive transfer of eosinophils was an increase in airways reactivity to i.v. β-methylcholine. Interestingly, airways hyperreactivity of mice that received i.v. eotaxin (figure V.13a) was not as pronounced as those that received the i.v. adoptive transfer of eosinophils (figure V.13b and c), even though the BALF (figure V.10a) and tissue levels (figure V.11) of eosinophils in all of these treatment groups were equal.

MBP was detected in the BALF of all groups of sensitised IL-5$^{-/-}$ mice following aeroallergen provocation (data not shown). Although the BALF eosinophil levels and airways reactivity were significantly greater in the groups that received i.v. eotaxin or an adoptive transfer of eosinophils when compared to groups that received control vehicle i.v., there were no obvious increases in the level of MBP in BALF of any of these groups (data not shown). However, the inability to detect changes in MBP levels between treatment groups and their controls may reflect the low levels of eosinophils in the BALF.

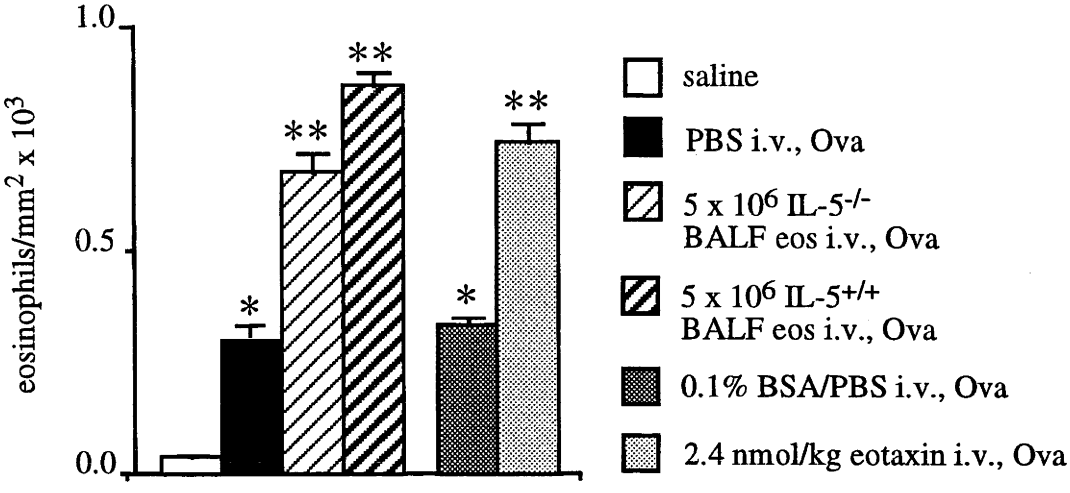


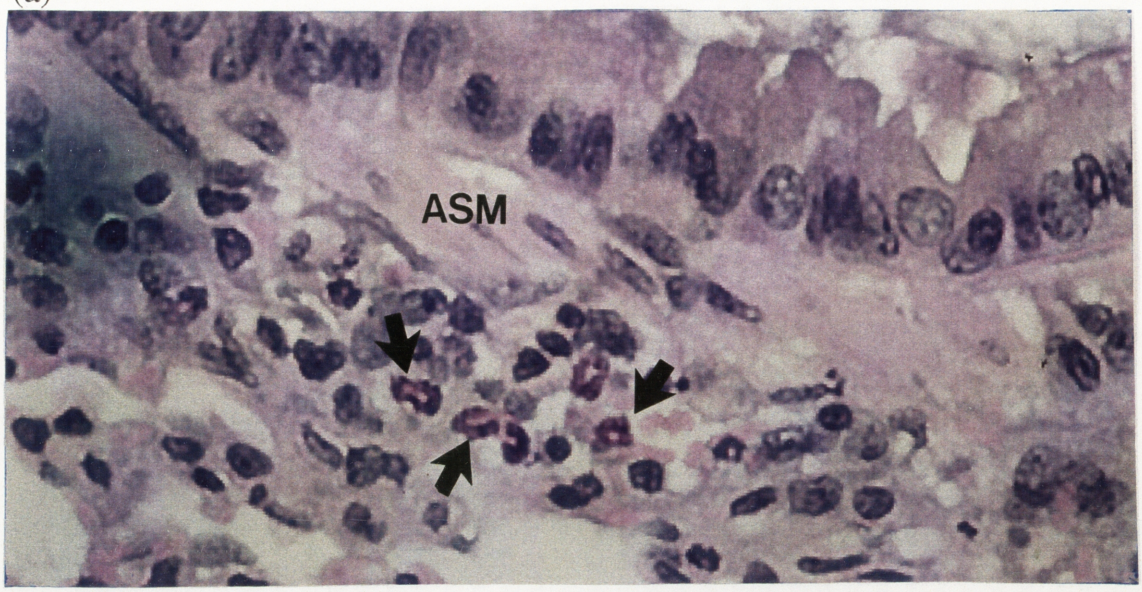
Figure V.11 ***The adoptive transfer of 5 x 10^6 donor eosinophils or the i.v. administration of eotaxin, significantly amplifies eosinophil levels in pulmonary tissues during allergic airways inflammation in IL-5$^{-/-}$ mice.***

IL-5$^{-/-}$ mice were sensitised to Ova by i.p injection on days 0 and 12 and exposed to Ova aerosol on days 24, 26, 27 and 28. On day 27 some mice received an adoptive transfer of 5 x 10^6 donor eosinophils that were isolated from either the BALF of allergic IL-5$^{+/+}$ mice, the BALF of allergic IL-5$^{-/-}$ mice inoculated with VV-HA-IL-5 or the blood of IL-5$^{-/-}$ mice inoculated with VV-HA-IL-5. Mice were injected i.v. with eotaxin (2.4 nmol/kg) or control vehicle [10 mM PBS/0.1% BSA (pH 7.4)] instead of an adoptive transfer of eosinophils. Eosinophil/mm^2 of lung sections (formalin fixed, paraffin embedded and stained with Haematoxylin and Eosin) were quantified on day 30. Data represents mean eosinophils/mm^2 in peribronchialar regions near airways smooth muscle ± SEM of groups of 3 mice. At least 10 fields of view were counted/lung section. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Difference in means were considered significant if $P < 0.05$. $*P < 0.001$ compared with non-sensitised IL-5$^{-/-}$ mice exposed to saline aerosol (saline) and $**P < 0.05$ when compare with control vehicle i.v. (PBS for eosinophil recipients and PBS/0.1% BSA for i.v. eotaxin injected control groups).

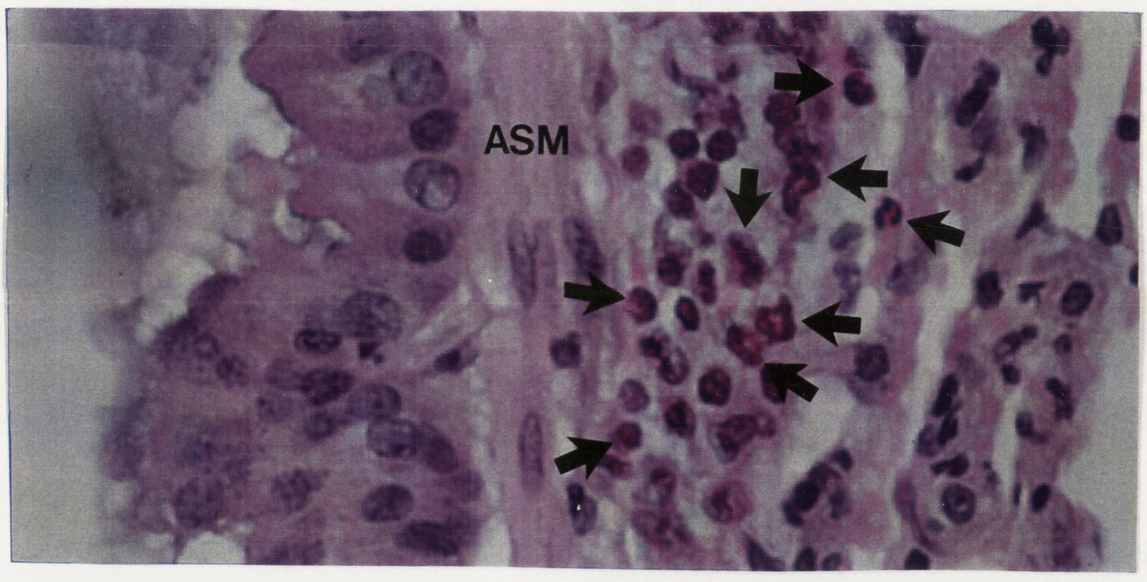
Figure V.12 ***Eosinophils specifically localise to regions near airways smooth muscles during allergic airways inflammation.***

Eosinophils that infiltrated the lungs of IL-$5^{-/-}$ mice during allergic airways inflammation on day 29 were specifically localised in peribronchial regions near the airways smooth muscle. (a) Significant levels of eosinophils were found in regions near the airways smooth muscle following aeroallergen provocation and (b) these levels were significantly increased in groups that received an i.v. adoptive transfer of eosinophils or an i.v administration of eotaxin (not shown). (c) No eosinophils were found in this region in the non-sensitised control groups. Photos are of 8 μM formalin-fixed paraffin sections stained with Haematoxylin and Eosin (400 x magnification).

(a)



(b)



(c)

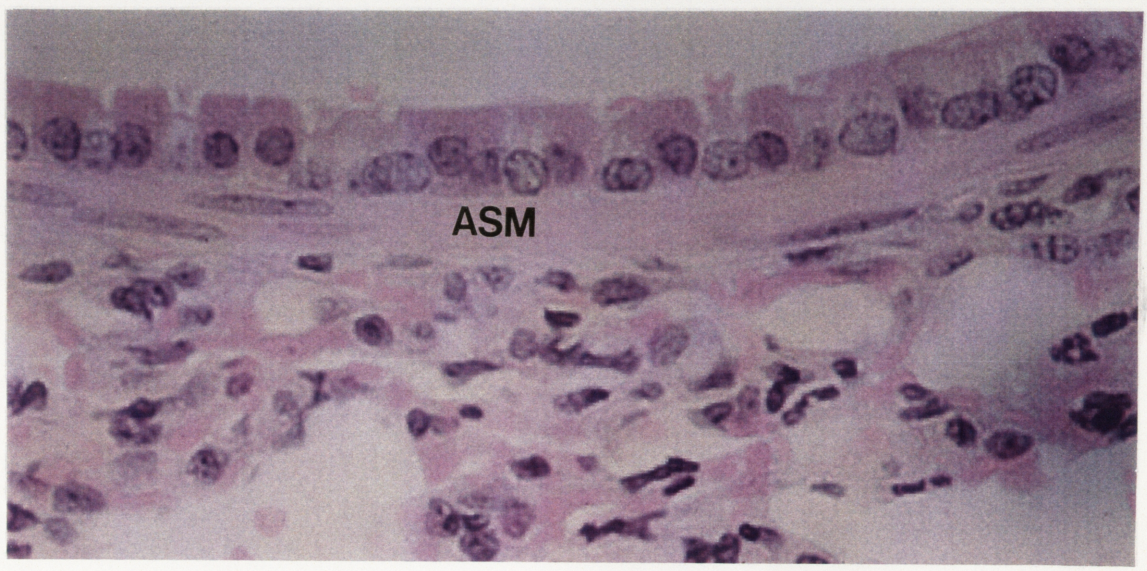
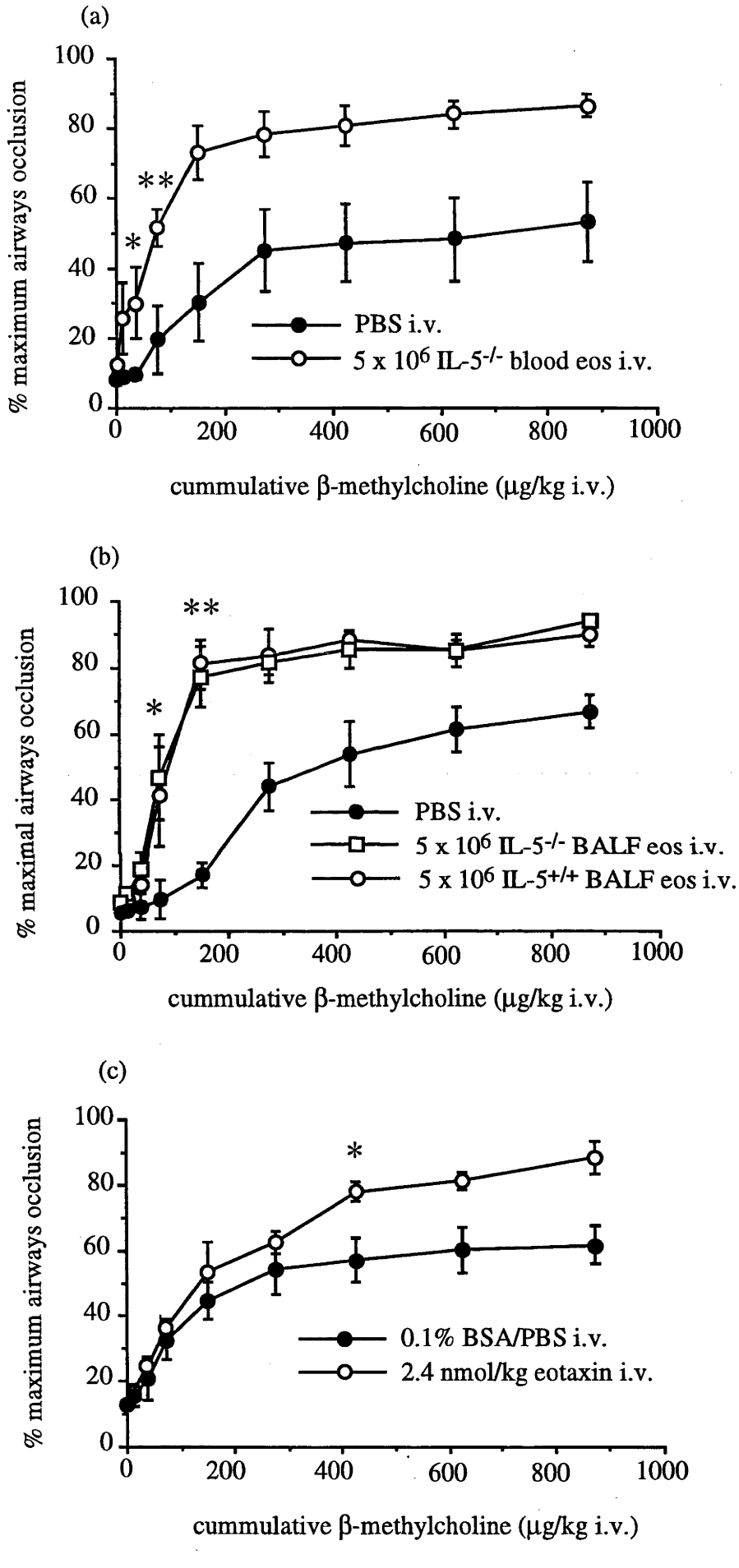


Figure V.13 ***The adoptive transfer of 5 x 10^6 donor eosinophils or the i.v. administration of eotaxin, significantly amplifies airways hyperreactivity during allergic airways inflammation in IL-$5^{-/-}$ mice.***

IL-$5^{-/-}$ mice were sensitised to Ova by i.p injection on days 0 and 12 and exposed to Ova aerosol on days 24, 26, 27 and 28. On day 27 some mice received an adoptive transfer of 5 x 10^6 donor eosinophils that were isolated from either the BALF of allergic IL-$5^{+/+}$ mice, the BALF of allergic IL-$5^{-/-}$ mice inoculated with VV-HA-IL-5 or the blood of IL-$5^{-/-}$ mice inoculated with VV-HA-IL-5. Another group of mice were injected i.v. with eotaxin (2.4 nmol/kg) or control vehicle [10 mM PBS/0.1% BSA (pH 7.4)] instead of an adoptive transfer of eosinophils. Airways hyperreactivity to i.v. β-methylcholine was measured on day 29, 24 hours after the final exposure to Ova aerosol. The lungs of the sensitised IL-$5^{-/-}$ mice that received an i.v. adoptive transfer of 5 x 10^6 eosinophils isolated from either (a) the blood of IL-$5^{-/-}$ mice inoculated with VV-HA-IL-5 or (b) the allergic BALF of IL-$5^{+/+}$ mice or the allergic BALF of IL-$5^{-/-}$ mice inoculated with VV-HA-IL-5 displayed airways reactivity to i.v. β-methylcholine compared with groups that received i.v. PBS only. (c) The groups that received i.v. eotaxin (2.4 nmol/kg) on day 27 also displayed airways hyperreactivity to i.v. β-methylcholine on day 29 when compared with a group that received i.v. control vehicle (PBS/0.1% BSA) only, although the elevations in airways reactivity were observed at a higher dose of β-methylcholine and were not as significant as that induced by the adoptive transfer of eosinophils. Airways reactivity was measured by monitoring i.v. β-methylcholine-induced changes in respiratory overflow volume. Data represents mean percentage airway occlusion ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) $*P < 0.05$ and $**P < 0.02$ when compared with PBS i.v at the same dose of β-methylcholine. (b) $*P < 0.05$ and $**P < 0.002$ when compared with PBS i.v at the same dose of β-methylcholine and (c) $*P < 0.05$ when compared with PBS/0.1% BSA i.v. at the same dose of β-methylcholine.


(a)
100
80
60
40
20
0
% maximum airways occlusion
*
**
PBS i.v.
5 x 10^6 IL-5^-/- blood eos i.v.
0
200
400
600
800
1000
cummulative β-methylcholine (μg/kg i.v.)
(b)
100
80
60
40
20
0
% maximal airways occlusion
*
**
PBS i.v.
5 x 10^6 IL-5^-/- BALF eos i.v.
5 x 10^6 IL-5^+/+ BALF eos i.v.
0
200
400
600
800
1000
cummulative β-methylcholine (μg/kg i.v.)
(c)
100
80
60
40
20
0
% maximum airways occlusion
*
0.1% BSA/PBS i.v.
2.4 nmol/kg eotaxin i.v.
0
200
400
600
800
1000
cummulative β-methylcholine (μg/kg i.v.)

V.4 DISCUSSION.

It is clear from studies in IL-5 deficient mice that this cytokine has an important role in the maintenance of basal circulating eosinophil levels (Kopf *et al.*, 1996). Phenotypic observations of mice genetically altered for the over production of IL-5 also support a role for this cytokine in promoting eosinophil differentiation and the release of eosinophils from bone marrow stores into the circulation (Dent *et al.*, 1990; Tominaga *et al.*, 1991). In studies presented in Chapter II, an essential requirement for IL-5 in eosinophil trafficking in response to eotaxin was demonstrated. Collectively, these observations [and many others (Foster *et al.*, 1996; Mould *et al.*, 1997; Lee *et al.*, 1997)] suggests an important role for IL-5 in mechanisms that regulate eosinophil trafficking.

V.4.1 Role of IL-5 in the homing of eosinophils to sites of allergy in the skin and lung.

Despite the indication that IL-5 may play a significant role in regulating the accumulation of eosinophils in inflamed tissues during allergy, the role for this cytokine in the homing mechanisms of eosinophils to the skin or lung during allergic inflammation remains unknown. Similar to the studies by Iwamoto *et al.*, (1994) (which involved the use of anti-IL-5 mAb), a reduction in the accumulation of eosinophils was observed at sites of CLPR at 6 and 24 hours in IL-$5^{-/-}$ mice relative to wild types. Notably, sensitised IL-$5^{-/-}$ mice had significantly lower levels of eosinophils in the circulation than sensitised wild types (figure V.3b). Eosinophilic responses to s.c. control vehicle were also reduced in the IL-$5^{-/-}$ mice when compared with the IL-$5^{+/+}$ mice (figure V.3a). Thus, the impaired eosinophilic responses in the skin of IL-$5^{-/-}$ mice may have been attributable to a reduced recruitable eosinophil pool in the blood. Notably, adoptively transferred eosinophils homed to sites of CLPR in the IL-$5^{-/-}$ and IL-$5^{+/+}$ mice groups with the same efficiency, demonstrating that IL-5 was not directly required for eosinophil homing during the first or second phase of eosinophil accumulation at sites of CLPR. In contrast, studies by Iwamoto *et al.*, (1992) suggests that the second phase of eosinophil accumulation at sites of CLPR, but not the first phase are dependent on IL-5. IL-5 may act to prime the eosinophils in the circulation for enhanced chemotactic responses and/or may amplify the circulating eosinophil pool by promoting the release of eosinophils from bone marrow (see Chapter II). The pretreatment of mice with anti-IL-5 mAb may decrease the second phase of eosinophil recruitment at sites of CLPR by inhibiting these processes.

It is interesting to note that IL-$5^{+/+}$ mice, but not IL-$5^{-/-}$ mice, have significantly higher levels of eosinophils in the circulation following i.p. sensitisation to Ova. The induction of peripheral eosinophilia in wild type mice may be due to the higher levels of IL-5 in sera

(Ohkawara *et al.*, 1997), which stimulates eosinophil differentiation in the bone marrow and mobilisation into the blood.

Similar to studies in the skin, no obligatory role for IL-5 in eosinophil homing to the allergic lung was demonstrated. Adoptively transferred eosinophils migrated to the allergic airways of both IL-$5^{-/-}$ and IL-$5^{+/+}$ mice. Interestingly, IL-$5^{-/-}$ mice poorly developed airways eosinophilia during allergic airways inflammation in contrast to wild types, even though homing responses are not reduced. Thus, similar to the observations in mice during CLPR, the reduced accumulation of eosinophils in the airways of IL-$5^{-/-}$ mice during allergic airways inflammation may reflect the reduced circulating eosinophil levels in these mice as well as the inability of these mice to develop blood eosinophilia. Collectively, these data suggest that the primary role of IL-5 may be to regulate the development of blood eosinophilia during allergic inflammation (Foster *et al.*, 1996).

V.4.2 Role of IL-5 in the development of airways hyperreactivity.

Mice that are genetically altered for the specific over production of IL-5 in airway epithelial cells display pulmonary eosinophilia and airways hyperreactivity (Lee *et al.*, 1997). This indicates that IL-5 is not only involved in the activation of eosinophil recruitment pathways, but also in the development of airways hyperreactivity, possibly though the activation of eosinophils. Although previous reports (Foster *et al.*, 1996) have indicated that sensitised IL-$5^{-/-}$ mice fail to develop airways hyperreactivity following exposure to aeroallergen, in this study sensitised and Ova challenged IL-$5^{-/-}$ mice developed airways hyperreactivity (albeit markedly reduced) to i.v. β-methylcholine during allergic airways inflammation. Sensitised IL-$5^{-/-}$ mice also exhibited an elevation in the level of eosinophils in the airways following aeroallergen exposure, however, this response was also markedly reduced in comparison to wild type mice. The degree of airways hyperreactivity in IL-$5^{+/+}$ and IL-$5^{-/-}$ mice also correlated with the level of eosinophils, and the presence of free MBP in the BALF. Collectively, these observations suggest a role for eosinophils and MBP in the induction of airways hyperreactivity independently of IL-5 during allergic airways inflammation.

Further evidence that eosinophils are responsible for the induction of airways hyperreactivity was shown in the temporal development of enhanced reactivity during allergic airways inflammation. Airways hyperreactivity developed more rapidly in IL-$5^{+/+}$ mice than in IL-$5^{-/-}$ mice and directly correlated with the degree of pulmonary eosinophilia. Interestingly, there also appears to be a delay between the accumulation (and activation) of eosinophils in BALF and the development of airways hyperreactivity in mice. Sensitised IL-$5^{+/+}$ mice did not exhibit airways hyperreactivity 24 hours after the initial aeroallergen exposure, even though these mice had levels of eosinophils in the BALF that were equivalent to that observed in IL-$5^{-/-}$ mice following multiple exposures

of aeroallergen and when IL-5$^{-/-}$ mice exhibited airways hyperreactivity. Furthermore, MBP was detectable in the BALF of the sensitised IL-5$^{+/+}$ mice 24 hours after the first aeroallergen challenge. Thus, other factors associated with the development of allergic inflammation (perhaps T-cell factors) are required to regulate eosinophil-induced airways hyperreactivity.

The i.v. administration of eotaxin and the i.v. adoptive transfer of eosinophils to IL-5$^{-/-}$ mice selectively enhanced airways eosinophilia during allergic airways inflammation. This response may have involved the ability of both of these treatments to enhance the circulating eosinophil pool. Interestingly, the i.v. administration of eotaxin to sensitised IL-5$^{-/-}$ mice during an allergic reaction in the lungs was not as effective at inducing blood eosinophilia as this factor was in naive mice (chapter IV). The apparent decreased ability of i.v eotaxin to mount a blood eosinophilia in sensitised IL-5$^{-/-}$ mice may reflect a competitive sequestration of eosinophils from the circulation into inflamed tissues. Although i.v. eotaxin was not as effective at inducing a rapid blood eosinophilia in sensitised IL-5$^{-/-}$ mice compared to the i.v. adoptive transfer of 5 x 10^6 eosinophils, it still effectively amplified pulmonary eosinophilia in these animals. Moreover, both treatments resulted in the recruitment of approximately the same number of eosinophils into the airways.

Eosinophils isolated from sites of inflammation reportedly have a different phenotype from those isolated from blood (Hansel *et al.*, 1991). Pulmonary eosinophils express high levels of ICAM-1 and may have different activational states compared with this cell in the blood. However, the eosinophils that were isolated from the BALF and the peripheral blood homed with equal efficiency to the inflamed lungs of IL-5$^{-/-}$ mice. This indicates that if there are any phenotypic differences between these two eosinophil populations it does not effect the ability of these cells to home to sites of inflammation. Moreover, these results demonstrate that eosinophils that have already homed to sites of allergic inflammation remain mobile and are able to migrate to other sites of inflammation from the circulation. Thus, the accumulation of eosinophils at sites of inflammation may not be terminal and these cells may have the potential to migrate to other tissues following the resolution of inflammation.

Similar to previous observations in Chapter III, the specific localisation of eosinophils in regions proximal to the airways smooth muscle, rather than the airways epithelium, suggests that these cells (or their products) mediate the induction of airways hyperreactivity by acting on the airways smooth muscle. As previously stated, this process may involve the release of MBP and the antagonism of inhibitory M2 muscarinic receptors (Gleich *et al.*, 1995, Evans *et al.*, 1997).

on cholinergic nerves in and around airways smooth muscle.

Interestingly, the source of the donor cells used for adoptive transfer studies had no effect on the ability of eosinophils to home to pulmonary sites of allergic inflammation or to exacerbate airways hyperreactivity in sensitised IL-$5^{-/-}$ mice. In contrast, although i.v. eotaxin was as effective at amplifying airways eosinophilia in sensitised IL-$5^{-/-}$ mice as the adoptive transfer of eosinophils, it did not induce the same degree of airways hyperreactivity. This may reflect priming of donor eosinophils with IL-5 for enhanced degranulation as opposed to the i.v. eotaxin treatment where IL-5 was absent. Although the ability of eosinophils to induce airways hyperreactivity during allergy was not solely dependent on IL-5 priming, it was markedly enhanced by it. Interestingly, the ability of the adoptively transferred eosinophils to produce their own IL-5 (IL-$5^{+/+}$ cells) had no observable effect on the ability of these cells to home to the inflamed lungs of sensitised IL-$5^{-/-}$ mice or to induce airways hyperreactivity.

These experiments demonstrate that eosinophils can home to the allergic lungs of mice in the absence of IL-5. Furthermore, they have shown for the first time that eosinophils can regulate airways hyperreactivity independently of IL-5. However, IL-5 plays a central role in enhancing eosinophil function or eosinophil-induced airways hyperreactivity. In light of the observation that eosinophils specifically localise to regions proximal to the airways smooth muscle it is hypothesised that this cell effects changes in airways function via interaction with this cell.

These experiments implicate the eosinophil as a central mediator of airways hyperreactivity associated with allergic airways inflammation. Airways hyperreactivity is a hallmark of asthma and our results endorse the eosinophil as a primary target for the design of novel therapeutics. Furthermore, since IL-5 plays a key role in the amplification of eosinophil-mediated disease pathology, the inhibition of this cytokine may also be useful for the treatment for allergic diseases.

CHAPTER VI

THE ROLE OF EOTAXIN IN EOSINOPHIL TRAFFICKING AND FUNCTION *IN VIVO*

VI.1 INTRODUCTION.

The increased expression of eotaxin at sites of allergic inflammation and its selective role in regulating eosinophil chemotaxis suggests that this chemokine may promote eosinophil recruitment into inflamed allergic tissues. Furthermore, a role for eotaxin in eosinophil trafficking during allergy has previously been demonstrated. The pretreatment of sensitised mice with anti-eotaxin mAb reduced aeroallergen-induced pulmonary eosinophilia by 50% (Gonzalo *et al.*, 1996). Similarly, mice rendered deficient in eotaxin by targeted disruption of the gene have impaired eosinophilic responses during early, but not the late phases of allergic airways inflammation (Rothenberg *et al.*, 1996). Earlier studies (Chapter II) showed that eotaxin potently induced a blood eosinophilia when administered i.v. to mice suggesting a role for this chemokine in regulating the circulating eosinophil pool. Recent reports also support a role for eotaxin in the regulation of blood eosinophil levels (Rothenberg *et al.*, 1996). Basal circulating levels of eosinophils in eotaxin deficient mice (eotaxin$^{-/-}$) were significantly reduced compared to wild types. Furthermore, the reduced accumulation of eosinophils at sites of inflammation in eotaxin$^{-/-}$ mice during the early phases of allergy may reflect these lower circulating eosinophil levels. The *in vitro* chemotactic activity of BALF from naive and allergic guinea pigs are also inhibited in the presence of anti-eotaxin mAb (Humbles *et al.*, 1997). Collectively, these studies suggest that eotaxin may regulate eosinophil trafficking at the basal state and during allergic responses.

There is an increase in the levels of eosinophils in the circulation of sensitised mice between 3 and 12 hours following aeroallergen provocation (Chapter V). Eotaxin mRNA is expressed at sites of allergic inflammation during this period indicating that eotaxin may play a role in inducing blood eosinophilia. It is likely that IL-5 plays a co-operative role with eotaxin in regulating blood eosinophilia in allergic mice. In contrast to naive IL-5$^{-/-}$ mice (Chapter II), i.v. administration of eotaxin after aeroallergen challenge does not induce as pronounced a blood eosinophilia (Chapter V). However, i.v. eotaxin did amplify the accumulation of eosinophils into the allergic lungs of IL-5$^{-/-}$ (Chapter V). Since eosinophils are actively sequestered by the allergic lung, the blood eosinophilic response normally induced by eotaxin is probably masked in sensitised IL-5$^{-/-}$ mice.

Eotaxin is constitutively expressed in numerous tissues that are commonly associated with eosinophils including the lungs, skin and intestines (Rothenberg *et al.*, 1995a, 1995b). Interestingly, mRNA for eotaxin is also expressed in a number of tissues that are not associated with eosinophils such as the thymus and lymph nodes. The expression of eotaxin in these two tissues suggested that it may signal to lymphocytes (Rothenberg *et al.*, 1995b). Recently, eotaxin was shown to induce intracellular calcium

signalling and the chemotaxis of a subclass of Th_2 lymphocytes. Furthermore, these Th_2 cells were shown to express CCR3 (Sallusto *et al.*, 1997). These observations are particularly interesting considering that eosinophil recruitment at sites of allergic inflammation is $CD4^+$ dependent (Iwamoto *et al.*, 1992; Gavett *et al.*, 1994). Thus, eotaxin may have a range of actions on specific leukocyte subsets.

Th_2-type immune responses, particularly those involving $CD4^+$ T-cells are thought to underlie the pathogenesis of allergic airways disease (Robinson *et al.*, 1992; Bochner *et al.*, 1994). Interestingly, the expression of eotaxin at sites of allergic airways inflammation in mice is not affected in the absence of CD4 (Gonzalo *et al.*, 1996), although it is dependent on the presence of $CD3^+$ lymphocytes (Maclean *et al.*, 1996). This suggests that $CD4^+$ cells do not primarily regulate eotaxin expression during allergy. $CD3^+$ lymphocytes may regulate eotaxin expression through the secretion of cytokines, however, the modulatory mechanism is currently unknown.

Eosinophil activation and the induction of airways hyperreactivity in the presence of eotaxin, IL-5 and antigen presentation is CD4-dependent (Chapter III). Furthermore, there was a correlation between the levels of lymphocytes and eosinophils in BALF and the induction of airways hyperreactivity during allergic airways inflammation in mice (Chapter V). In light of the demonstration that both eosinophils and Th_2-lymphocytes express the eotaxin receptor (CCR3) (Heath *et al.*, 1997; Sallusto *et al.*, 1997), eotaxin may have an important role in the activation of these cell types at sites of allergic inflammation.

Although numerous studies have investigated the role of eotaxin in eosinophil recruitment at sites of allergic inflammation, the role of this chemokine in the aetiology of airways damage and hyperreactivity has not been determined. Eotaxin was recently shown to potently induce respiratory bursts in human eosinophils and in this capacity was comparable to the complement peptide, C5a (Elsner *et al.*, 1996). Other C-C-chemokines, including RANTES and MCP-3 also induce respiratory bursts in eosinophils, although eotaxin appears to be the most potent.

In this Chapter the role of eotaxin in the trafficking of eosinophils was investigated in eotaxin deficient mice ($eotaxin^{-/-}$). The role of eotaxin in regulating blood eosinophilia in naive mice and in the mechanisms of eosinophil recruitment and activation at sites of allergic inflammation was investigated. In addition, the expression of eotaxin mRNA in the lungs and skin of sensitised IL-5, IL-4 and IFN-γ deficient mice following antigen provocation was measured.

VI.2 MATERIALS AND METHODS.

VI.2.1 Analysis of the effect of eotaxin deficiency on eosinophil trafficking in the blood and eosinophil mobilisation in bone marrow.

Eotaxin deficient mice (eotaxin$^{-/-}$) were a gift from Professor Mark Rothenberg (Childrens Hospital Medical Centre, Cincinnati, OH., USA.). To determine the requirement of eotaxin for the regulation of eosinophil levels in the circulation and the bone marrow eotaxin$^{-/-}$ and eotaxin$^{+/+}$ mice (129Sv$_{Ev}$, a gift from Dr Alistair Ramsay, John Curtin School of Medical Research, Australian National University) were injected i.v. with IL-5 (100 pmol/kg) and/or eotaxin (1.2 nmol/kg) or control vehicle [100 µl 0.1%BSA/PBS (pH 7.4)] and the levels of eosinophil in these two compartments monitored. Blood samples were taken immediately prior to and at 30 minutes post i.v. injection and eosinophils/ml of blood were determined using Discombe's method (see section II.2.2). The mice were sacrificed by cervical dislocation 30 minutes after i.v. injections and the levels of eosinophils in the bone marrow of the femurs (right and left) were quantified (see section II.2.3).

Eotaxin$^{-/-}$ and eotaxin$^{+/+}$ mice were also injected i.v. with eotaxin (1.2 nmol/kg) or IL-5 (100 pmol/kg) and the levels of eosinophils in the circulation determined every 30 minutes for a period of 3 hours. Blood samples taken via the tail vein and eosinophils/ml of blood were determined using Discombe's method (see section II.2.2).

VI.2.2 The effect of IL-5 on primary cultures of bone marrow cells from eotaxin$^{-/-}$ mice.

Bone marrow cells were obtained from two eotaxin$^{-/-}$ and two eotaxin$^{+/+}$ mice by lavage with 2 mls of HBSS (see section II.2.3). Nucleated cells were pelleted by centrifugation (400 x g for 5 mins at 4°C) and resuspended to 1 x 10^6 cells/ml in mixed lymphocyte culture medium (MLC)/10% heat inactivated FCS. MLC consisted of DMEM supplemented with 4 mg/ml folic acid, 66 µg/ml L-asparagine, 116 µg/ml L-arginine-HCl, 2 mg/ml $NaHCO_3$, 2 mM L-glutamine, 1 mM sodium pyruvate and 10 mM HEPES. Cell viability was > 95% as determined by trypan blue exclusion. The cells were dispensed into 96 well tissue culture plates at 2 x 10^5 cells/well and IL-5 (in MLC/10% FCS) was added to half of the wells to give a final concentration of 30 nM. Control cultures received MLC/10% FCS only. The cultures were then incubated at 37°C/5% CO_2. Eosinophils as a percentage of total cells in culture were determined at 0, 0.5, 1, 2 and 3 hours after IL-5 administration. Briefly, the cells were gently removed from triplicate wells using a 1 ml pipette to avoid cell damage. This process recovered > 80% of the cells and the recovered cell viability (trypan blue exclusion) was > 92% after

3 hours of culture, irrespective of the treatment. The cell samples (1 x 10^5 cells) were cytocentrifuged onto glass slides and stained with Giemsa/May-Grunwald. Eosinophils (identified by morphological criteria), as a percentage of total leukocytes were quantified by routinely counting 200-300 total nucleated cells.

VI.2.3 Analysis of eotaxin expression at skin and lung sites of allergic inflammation by semi-quantitative reverse transcription-polymerase chain reaction.

VI.2.3.1 *Determination of peak eotaxin expression in the skin and lungs following allergen provocation using reverse transcription-polymerase chain reaction.*

VI.2.3.1.1 Preparation of samples and isolation of total RNA.

Mice (male C57BL6, 6-8 weeks of age) were sensitised to Ova by i.p injection on days 0 and 12 (see section V.2.1.1). Control non-sensitised animals received 1 mg Alhydrogel (CSL, Parkville, Melbourne, Australia) in saline only. On day 24, some of the sensitised mice were exposed to an aerosol of Ova (10 mg/ml) in 0.9% saline for 1 hour while others received a s.c. injection of 50 µg of Ova in 0.1 mls of saline (see section V.2.1.1). The non-sensitised control groups were exposed to an aerosol of saline or received a s.c. injection of 0.1 mls of saline. At 0, 0.5, 1, 2, 3, 6, 12 and 24 hours post Ova or saline exposure the mice were sacrificed by CO_2 asphyxiation and the lungs (mice exposed to aerosolized Ova or saline) or the s.c. membrane (mice given s.c. injections) removed and immediately placed into 10 volumes of RNAzol B (BIOTEX Laboratories, Houston, Texas, USA.) on ice. The samples were homogenised and the RNA extracted using RNAzol B according to the manufacturer's instructions. RNA samples were stored in RNase-free water at -70°C.

VI.2.3.1.2 Reverse transcription-polymerase chain reaction for eotaxin and hypoxanthine riboysltransferase.

RNA (1 µg) was transcribed to first strand cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI., USA.) according to manufactures instructions. Reaction mixture (20 µl) contained 1 µg of RNA, 25 mM Tris-HCl (pH 8.3), 37.5 mM KCl, 1.5 mM $MgCl_2$, 5 mM dithiothrietol, 25 µg/ml oligo dT_{15} and 10 mM each of dATP, dCTP, dGTP and dTTP.

VI.2.3.1.2.1 *Eotaxin polymerase chain reaction.*

cDNA (5 µl of 1/30 dilution of lung samples or 1/20 dilution of membrane samples all in PCR-grade water) was added to a 20 µl reaction mix containing 5 pmol of each of the forward primer (eotax F1) and reverse primer (eotax R1), 1 x AmpliTaq buffer (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ., USA.), 1 unit of AmpliTaq DNA polymerase (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ., USA.) and 250 µM each of dGTP, dCTP, dATP and dTTP (Promega, Madison, WI., USA,). To control for contamination, a water control was also included and consisted of 5 µl of PCR-grade water instead of 5 µl of sample.

The PCR primers, eotax F1 (5' TCCACCATGCAGAGCTCCACAG 3') and eotax R1 (5' CCCACATCTCCTTTCATGCCCC 3') were homologous to the 5' (42-63 bases) and 3' (complementary to 551-572 bases) regions of the murine eotaxin cDNA. The eotax F1 primer was synthesised with a 6-FAM fluorescent tag attached to the 5' end. The primers were synthesised by the Biomolecular Resource Facility at the John Curtin School of Medical Research (Australian National University) and were supplied in ammonium hydroxide. Prior to use the primers were vacuum dried, washed and resuspended in PCR-grade water to the desired concentration.

The PCR was run using a FTS-1 Thermal Sequencer (Corbett Research, Sydney, Australia) under the following conditions. Samples were denatured at 94°C for 3 minutes followed by 40 cycles of denaturation at 96°C for 5 seconds, annealing at 50°C for 5 seconds and extension at 72°C for 1 minute. The reaction was then concluded with a 5 minutes extension at 72°C.

VI.2.3.1.2.2 *Hypoxanthine riboyltransferase polymerase chain reaction.*

Hypoxanthine riboysltransferase (HPRT) was used as a house keeping gene for semi quantitation of eotaxin mRNA expression as previously described (Svetic *et al.,* 1991).

cDNA (5 µl of 1/80 dilution of lung samples and 1/20 dilution of membrane samples in PCR-grade water) was added to a 20 µl reaction mix containing 5 pmol of each of the forward primer (HPRT F1) and reverse primer (HPRT R1), 1 x AmpliTaq buffer (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ., USA.), 1 unit of AmpliTaq DNA polymerase (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ., USA.) and 250 µM each of dGTP, dCTP, dATP and dTTP (Promega, Madison, WI., USA.). To control for contamination, a water control was also included and consisted of 5 µl of PCR-grade water instead of 5 µl of sample.

The non-degenerate PCR primers HPRT F1 (5' HEX-GATTCAACTTGCGCTCATCTT AGGC 3') and HPRT R1 (5' GTTGGATACAGGCCAGACTTTGTTG 3') homologous to 5' (514-538 bases) and 3' (complementary to 652-678 bases) regions of the murine HPRT gene were previously designed (Svetic *et al.*, 1991). The primers were synthesised by the Biomolecular Resource Facility at the John Curtin School of Medical Research (Australian National University) and were supplied in ammonium hydroxide. HPRT F1 was synthesised with a 5' HEX fluorescent label. Prior to use the primers were vacuum dried, washed and resuspended in PCR-grade water to the desired concentration. The PCR was run using a FTS-1 Thermal Sequencer (Corbett Research, Sydney, Australia) under the following conditions. Samples were denatured at 94°C for 3 minutes followed by 40 cycles of denaturation at 96°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 2 minutes . The reaction was then concluded with a 5 minutes extension at 72°C.

VI.2.3.1.3 Analysis of polymerase chain reaction products.

HPRT and eotaxin PCR products (1 µl each) were combined and analysed by the Biomolecular Resource Facility of the John Curtin School of Medical Research (Australian National University). Briefly, the PCR products were electrophoresed through a 2% polyacrylamide gel and the fluorescence of individual DNA fragments quantified using the GeneScan Analysis 2.0.2 software package. Peaks of fluorescence corresponding to HEX (HPRT) and 6-FAM (eotaxin) emissions were detected at the expected PCR product sizes (165 and 533 bp respectively). Eotaxin levels were determined in relation to the levels of HPRT by dividing the peak fluorescent area of the eotaxin PCR product by peak fluorescent area of the HPRT PCR product.

The PCR products were also analysed by PAGE (see section III.2.1.6.2.1) for comparative purposes.

VI.2.3.2 ***Determination of the role of IL-4, IL-5 and IFNγ on eotaxin mRNA expression at skin and lung sites of allergic inflammation.***

IL-4$^{-/-}$, IL-5$^{-/-}$, IFNγ$^{-/-}$ and the corresponding wild type mice were analysed for eotaxin mRNA expression (see section VI.2.3), relative to HPRT mRNA expression at skin and lung sites of allergic inflammation at times of peak eotaxin expression in the tissues [previously determined in wild type mice (3 and 6 hours respectively, see section VI.2.3.1)].

VI.2.4 Eotaxin deficiency and eosinophilic responses during cutaneous late-phase reaction.

Eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice were sensitised to Ova by i.p injection on days 0 and 12 (see section V.2.1.1). Control groups received 1 mg of Alhydrogel (CSL, Parkville, Melbourne, Australia) in 0.2 mls of saline. On day 24 the sensitised mice received a s.c. injection of 50 μg of Ova plus 0.9 mls of air and the non-sensitised controls received a s.c. injection of 0.1 mls of saline and 0.9 mls of air. The accumulation of eosinophils at s.c. injection sites were quantified at 6 and 24 hours after s.c. injections (see section V.2.1.2). The levels of eosinophils in the blood and bone marrow of these mice were also determined at 0, 6 and 24 hours post s.c. injection (see sections II.2.2 and II.2.3, respectively).

VI.2.5 The effect of eotaxin deficiency on pulmonary eosinophilia and the induction of airways hyperreactivity during allergic airways inflammation.

Eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice were sensitised to Ova by i.p. injection on days 0 and 12 (see section V.2.1.1). Non-sensitised controls received 1 mg Alhydrogel in 0.2 mls of saline only. On days 24 and 26 the sensitised mice were exposed to Ova aerosol (10 mg/ml) in 0.9% saline for 3 x 30 minutes with 30 minute rests between exposures. The non-sensitised controls were exposed to saline aerosol only. On day 27, 24 hours after the last aerosol exposure, groups were analysed for airways reactivity to i.v. β-methylcholine (see section III.2.11). This was immediately followed by the quantitation of BALF leukocyte levels (see section III.2.9). Circulating leukocytes were quantified on blood smears (section V.2.1.4), prepared from samples taken on days 23, 25 and 27 (prior to the analysis of airways hyperreactivity).

VI.3 RESULTS.

VI.3.1 Eotaxin$^{-/-}$ mice display abnormal blood and bone marrow levels following i.v. administration of eotaxin and IL-5, respectively.

In contrast to previous reports (Rothenberg *et al.*, 1996), eotaxin$^{-/-}$ mice did not have reduced basal levels of eosinophils in the circulation when compared with eotaxin$^{+/+}$ mice (figure VI.1). The i.v. administration of eotaxin (1.2 nmol/kg), IL-5 (100 pmol/kg) or both of these cytokines together, induced a pronounced blood eosinophilia in eotaxin$^{-/-}$ and eotaxin$^{+/+}$ mice (129 Sv_{Ev} strain)(figure VI.1), supporting previous observations in C57BL/6 mice (see section II.3.1). The level of eosinophilia induced by IL-5 was similar in eotaxin$^{-/-}$ and eotaxin$^{+/+}$ mice (figure VI.1). In contrast, eotaxin (1.2 nmol/kg) induced a greater increase the level of eosinophils in the blood of eotaxin$^{-/-}$ mice than in the eotaxin$^{+/+}$ mice (figure VI.1). The i.v. coadministration of IL-5 (100 pmol/kg) and eotaxin (1.2 nmol/kg) significantly elevated the levels of eosinophils in the circulation of eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice above that induced by either of these cytokines alone (figure VI.1).

The i.v. administration of eotaxin to eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice did not affect the levels of eosinophils in the bone marrow when they were analysed at the peak of the blood eosinophilia. These results were similar to the observations of previous experiments in IL-5$^{+/+}$ and IL-5$^{-/-}$ mice (section IV.3.1). The i.v. administration of IL-5 (100 pmol/kg) to eotaxin$^{+/+}$ mice caused a significant decrease in the level of eosinophils in the bone marrow when measured at the peak of the blood eosinophilia (30 minutes post i.v. injection). Although eosinophilic responses to i.v. IL-5 in the blood were not affected in the absence of eotaxin (figure VI.1), there was an increase in the level of eosinophils in the bone marrow (figure VI.2). The i.v. coadministration of eotaxin and IL-5 did not alter the effect of IL-5 on eosinophil levels in the bone marrow of either the eotaxin$^{+/+}$ or eotaxin$^{-/-}$ groups (figure VI.2). In order to clarify the mechanism involved in the elevation in eosinophils levels in the bone marrow of eotaxin$^{-/-}$ mice following i.v. administration of IL-5, bone marrow cells were lavaged from the right femurs of eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice and were cultured with 30 nM rmIL-5 (a gift from Professor I. G. Young, John Curtin School of Medical Research, Australian National University) (figure VI.3). In the presence of IL-5, the levels of eosinophils in cultures of bone marrow cells from eotaxin$^{+/+}$ mice were unaffected over 3 hours (figure VI.3). In contrast, an increased proportion of eosinophils was observed in the cultures of bone marrow cells from eotaxin$^{-/-}$ mice (figure VI.3). Moreover, the increases in the percentage of eosinophils in these cultures were evident as early as 30 minutes after the

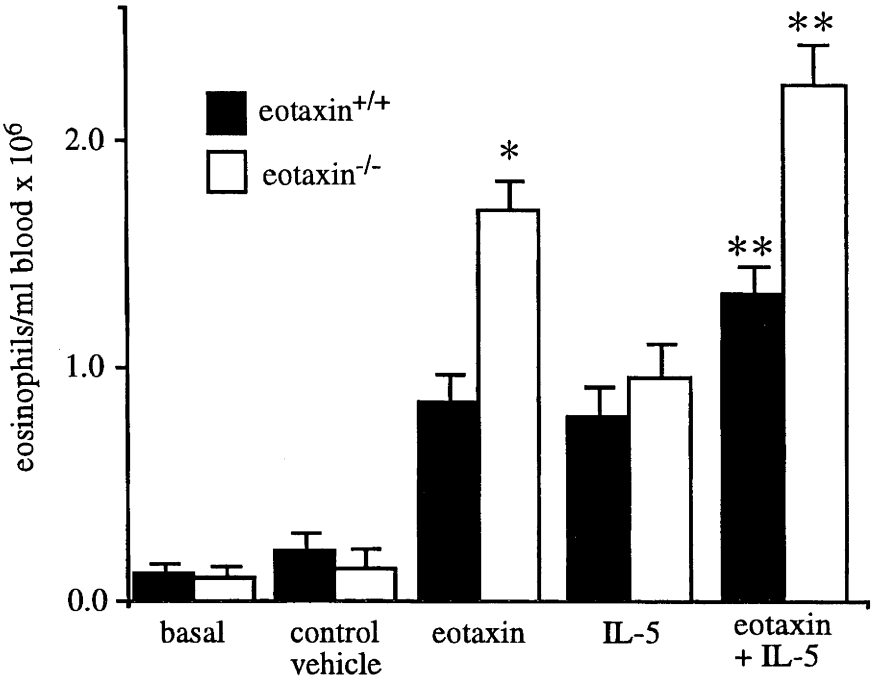


Figure VI.1 ***The effects of intravenous IL-5 and/or eotaxin administration on circulating eosinophil levels in mice in the absence of endogenous eotaxin.***

Eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice were injected i.v. with IL-5 (100 pmol/kg), eotaxin (1.2 nmol/kg), a combination of both of these cytokines or control vehicle [100 µl of 10 mM PBS/0.1% BSA (pH 7.4)]. Blood samples were taken before, at 30 minutes and hourly after i.v. injection of the cytokines or control vehicle for differential quantification of leukocytes (Data shown for 0 and 30 minutes only at peak of eosinophilia). The i.v. administration of IL-5 and/or eotaxin induced a significant increase in circulating eosinophil levels. Responses to i.v. eotaxin, but not i.v. IL-5 were further amplified in the eotaxin$^{-/-}$ mice. The i.v. coadministration of IL-5 and eotaxin induced a significant increase in circulating eosinophil levels that was significantly greater than that induced by these cytokines alone. Results represent mean eosinophils/ml of blood ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. $*P < 0.02$ compared with eotaxin$^{+/+}$ mice under the same conditions and $**P < 0.05$ compared with eotaxin or IL-5 alone in mice of the same genotype.

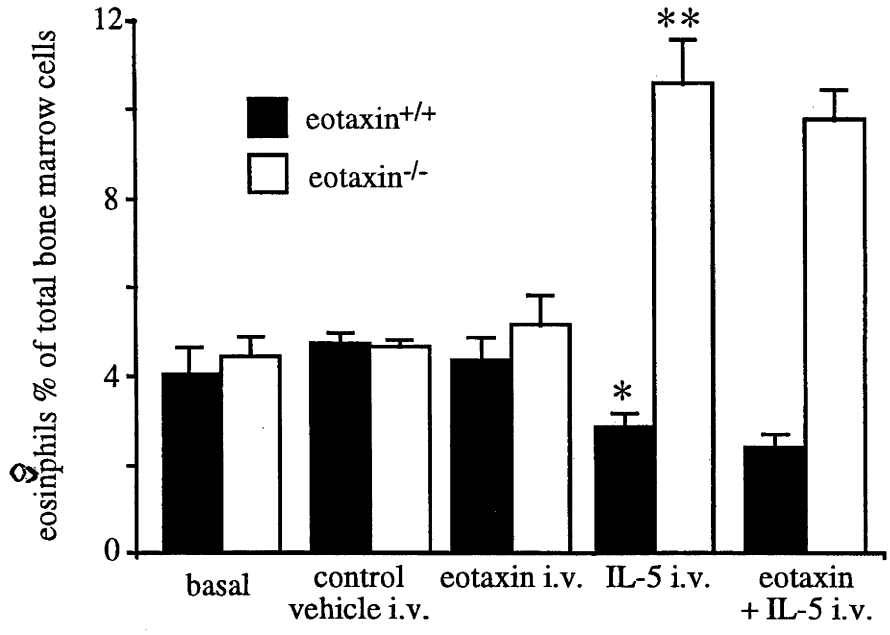


Figure VI.2 ***The effect of intravenous IL-5 and/or eotaxin administration on the level of eosinophils residing in bone marrow.***

Eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice were injected i.v. with IL-5 (100 pmol/kg), eotaxin (1.2 nmol/kg) or control vehicle [100 µl of 10 mM PBS/0.1% BSA (pH 7.4)]. Bone marrow eosinophil levels were quantified at the peak of the blood eosinophilia (30 minutes post i.v administration). Administration of i.v. IL-5 induced a significant decreased in the level of eosinophils in the bone marrow of the eotaxin$^{+/+}$ mice. In contrast, i.v IL-5 induced a significant increase in the level of eosinophils in the bone marrow of eotaxin$^{-/-}$ mice. The i.v. administration of eotaxin did not significantly effect the level of eosinophils in the bone marrow. Furthermore, eotaxin did not affect IL-5-induced mobilisation of eosinophils in either the eotaxin$^{-/-}$ or eotaxin$^{+/+}$ mice. Results represent mean percentage of eosinophils of total femur marrow cells of groups of 8 femurs from 4 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. * $P < 0.05$ compared with eotaxin$^{+/+}$ mice given control vehicle i.v and ** $P < 0.01$ compared with eotaxin$^{-/-}$ given control vehicle i.v. No significant differences were detected between means of the groups that received eotaxin plus IL-5 i.v and those that received IL-5 alone.

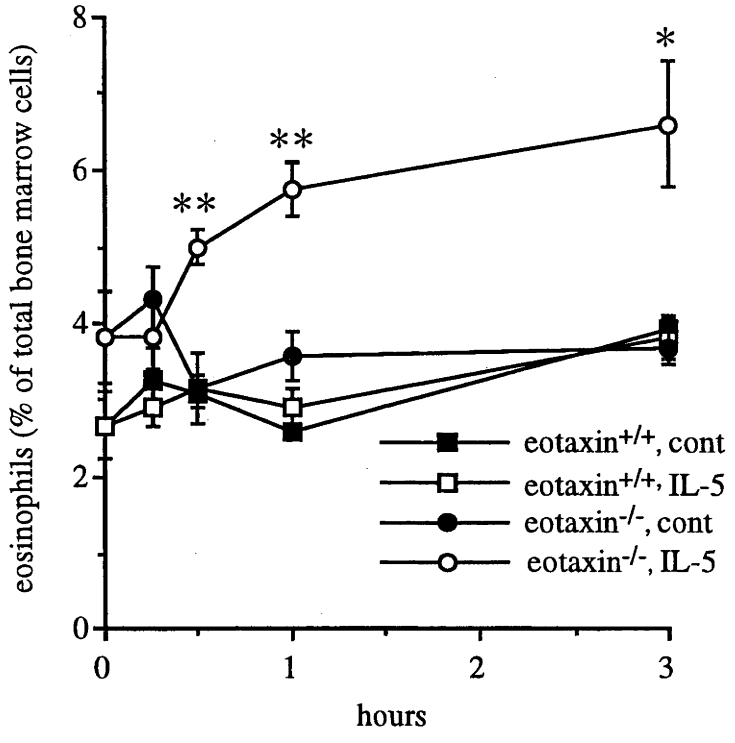


Figure VI.3 ***The culture of bone marrow cells isolated from eotaxin$^{-/-}$ mice with IL-5 leads to a rapid increase in eosinophil levels.***

Bone marrow cells obtained from the femurs of eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice were incubated with 30 nM rmIL-5. Elevated levels of eosinophils were detected in the bone marrow cultures from eotaxin$^{-/-}$ mice at 30 minutes after the addition of IL-5 but not in the control treatment. There were no changes in eosinophil levels in eotaxin$^{+/+}$ bone marrow cultures in the presence of IL-5 or in the control cultures for the duration of the experiment (3 hours). Results represent mean percentage of eosinophils of total bone marrow culture cells ± SEM. Bone marrow cells were obtained from 2 mice/group and were assayed in triplicate. The significance of differences between experimental groups were analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. $*P < 0.05$ when compared with either eotaxin$^{-/-}$ control, eotaxin$^{+/+}$ in the presence of IL-5 or the eotaxin$^{+/+}$ control groups at the same time or when compared with the eotaxin$^{-/-}$ cultures prior to IL-5 exposure. $**P < 0.02$ when compared with either , eotaxin$^{+/+}$ in the presence of IL-5 or the eotaxin$^{-/-}$ and eotaxin$^{+/+}$ control groups.

addition of the IL-5 (figure VI.3). This phenomena was specific for IL-5 since no changes in the proportion of eosinophils in the bone marrow cultures from eotaxin$^{+/+}$ or eotaxin$^{-/-}$ mice were observed in the control treatment (figure VI.3).

VI.3.2 Eotaxin mRNA expression is enhanced at sites of allergic inflammation and is differentially regulated at skin and lungs sites of allergic inflammation by IL-4 and IFNγ.

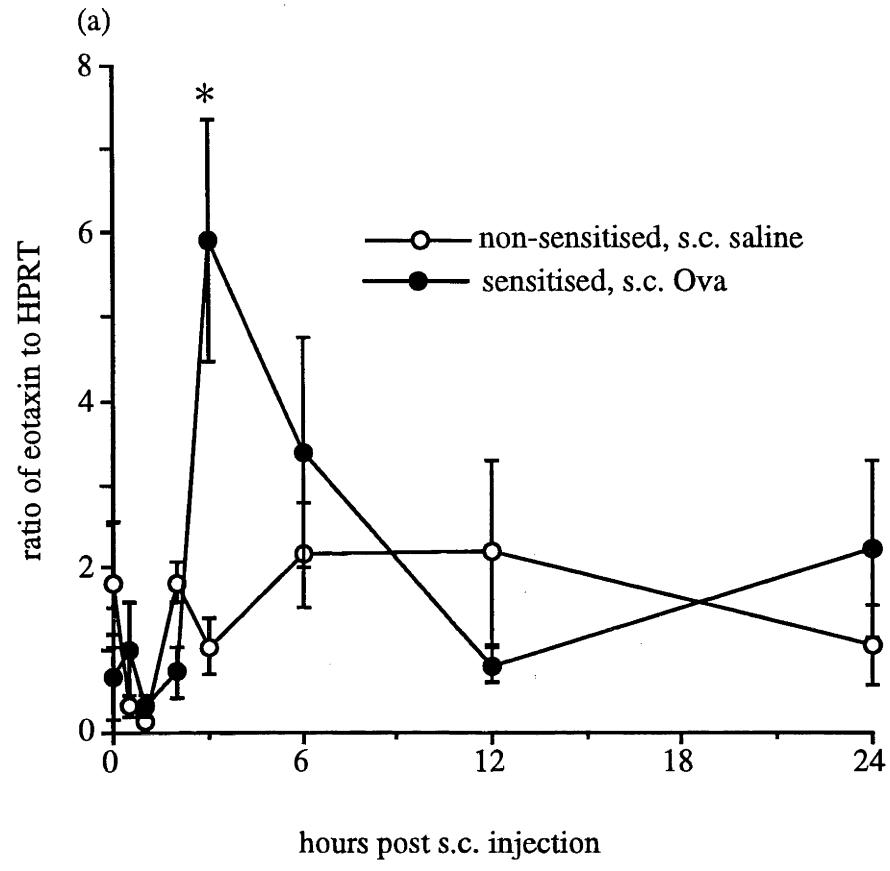
Eotaxin mRNA was quantified at skin and lung sites of allergic inflammation using semi-quantitative PCR analysis with reference to mRNA expression of the house keeping gene, HPRT (Svetic *et al.*, 1991). Eotaxin was constitutively expressed in the skin (figure VI.4a) and lungs (figure VI.4b) of antigen sensitised and non-sensitised mice. Similar observations have been reported in naive guinea pigs and mice (Rothenberg *et al.*, 1995a; 1995b).

Significant elevations in eotaxin mRNA expression were observed in the skin of the sensitised mice following the s.c. administration of Ova (figure VI.4a). No elevation in eotaxin mRNA expression was detected in the skin of the non-sensitised control mice following the s.c. administration of saline (figure VI.4a) suggesting that eotaxin does not promote non-specific eosinophils trafficking in response to saline administration. As previously observed in the allergic lungs of guinea pigs (Rothenberg *et al.*, 1995b) and mice (Gonzalo *et al.*, 1996b), the expression of eotaxin mRNA in the skin was enhanced soon after allergen provocation and this expression was transient (figure VI.4a). A significant elevation in eotaxin mRNA was detected at 3 hours following the s.c. administration of Ova, but not at any another time.

An elevation in the expression of eotaxin mRNA was also detected in the lungs of the sensitised mice following aeroallergen (Ova) challenge (figure VI.4b). In contrast, no elevation in eotaxin mRNA expression was detected in the non-sensitised mice following exposure to an aerosol of saline (figure VI.4b). As observed at sites of CLPR, the elevations in eotaxin mRNA expression in the allergic lungs of mice were detectable soon after aeroallergen exposure. Interestingly, in contrast to allergic skin and the previous reports of eotaxin mRNA expression in the allergic lungs of guinea pigs (Rothenberg *et al.*, 1995b) and mice (Gonzalo *et al.*, 1996b), the elevated expression of eotaxin mRNA in the allergic lungs was sustained. A significant elevation in the levels eotaxin mRNA in the lungs was first detected at 6 hours following the exposure to aeroallergen and the levels remained elevated for the duration of the experiment (24 hours) (figure VI.4b).

Figure VI.4 ***The kinetics of eotaxin expression at skin and lung sites of allergic inflammation in mice.***

Mice were sensitised to Ova by i.p. injection on days 0 and 12 and on day 24 cutaneous or airways inflammation was induced in sensitised mice by s.c. Ova administration or by Ova aerosol exposure, respectively. The non-sensitised controls received s.c. saline or were exposed to an aerosol of saline instead of Ova exposure. The levels of eotaxin mRNA at skin and lung sites was monitored over a 24 hours. Eotaxin mRNA expression was compared with HPRT mRNA expression and was determined using semi quantitative RT-PCR. (a) Eotaxin mRNA was maximally expressed at 3 hours post Ova administration in the skin of sensitised mice and fell thereafter. No significant increase in eotaxin mRNA expression relative to HPRT mRNA expression was detectable in non-sensitised mice in response to s.c. administration of saline. (b) Elevated levels of eotaxin mRNA were detectable in the lungs of sensitised mice 6 hours post Ova aerosol exposure and remained elevated for a further 18 hours. No significant increase in eotaxin mRNA expression relative to HPRT mRNA expression was detectable in the non-sensitised mice after exposure to saline aerosol. The significance of differences between experimental groups were analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) $*P < 0.02$ when compared with non-sensitised mice 3 hours post s.c. injection of saline. (b) $*P < 0.02$ when compared with non-sensitised mice 6 hours post exposure to saline aerosol.


(a)
8
6
4
2
0
*
ratio of eotaxin to HPRT
non-sensitised, s.c. saline
sensitised, s.c. Ova
0
6
12
18
24
hours post s.c. injection

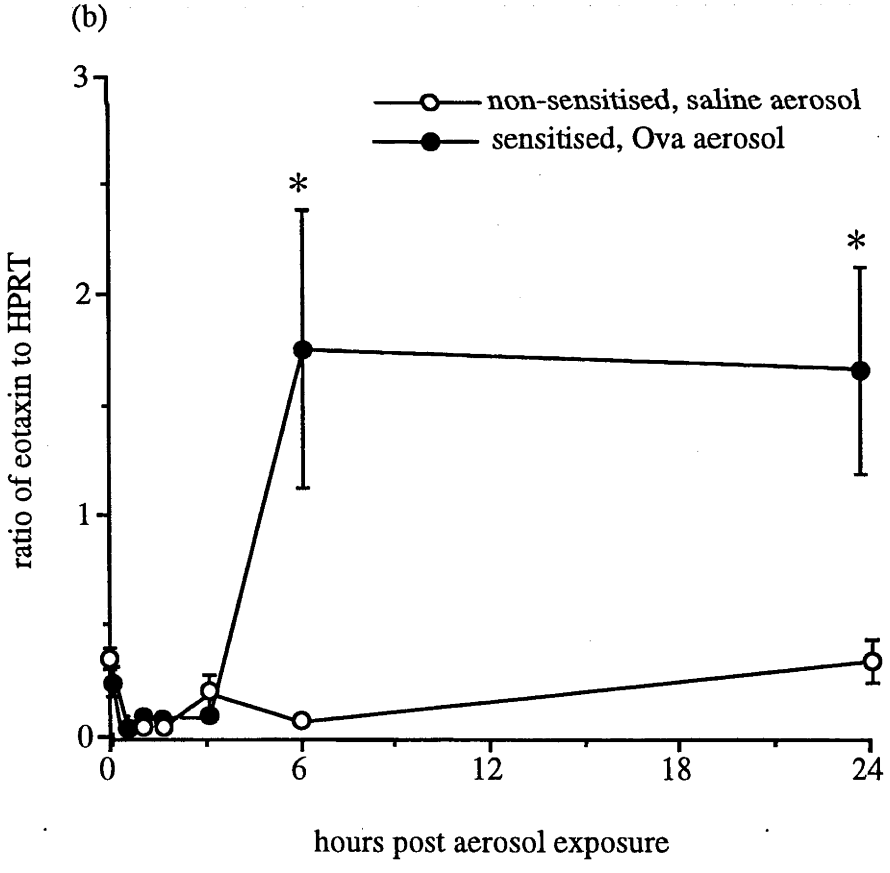
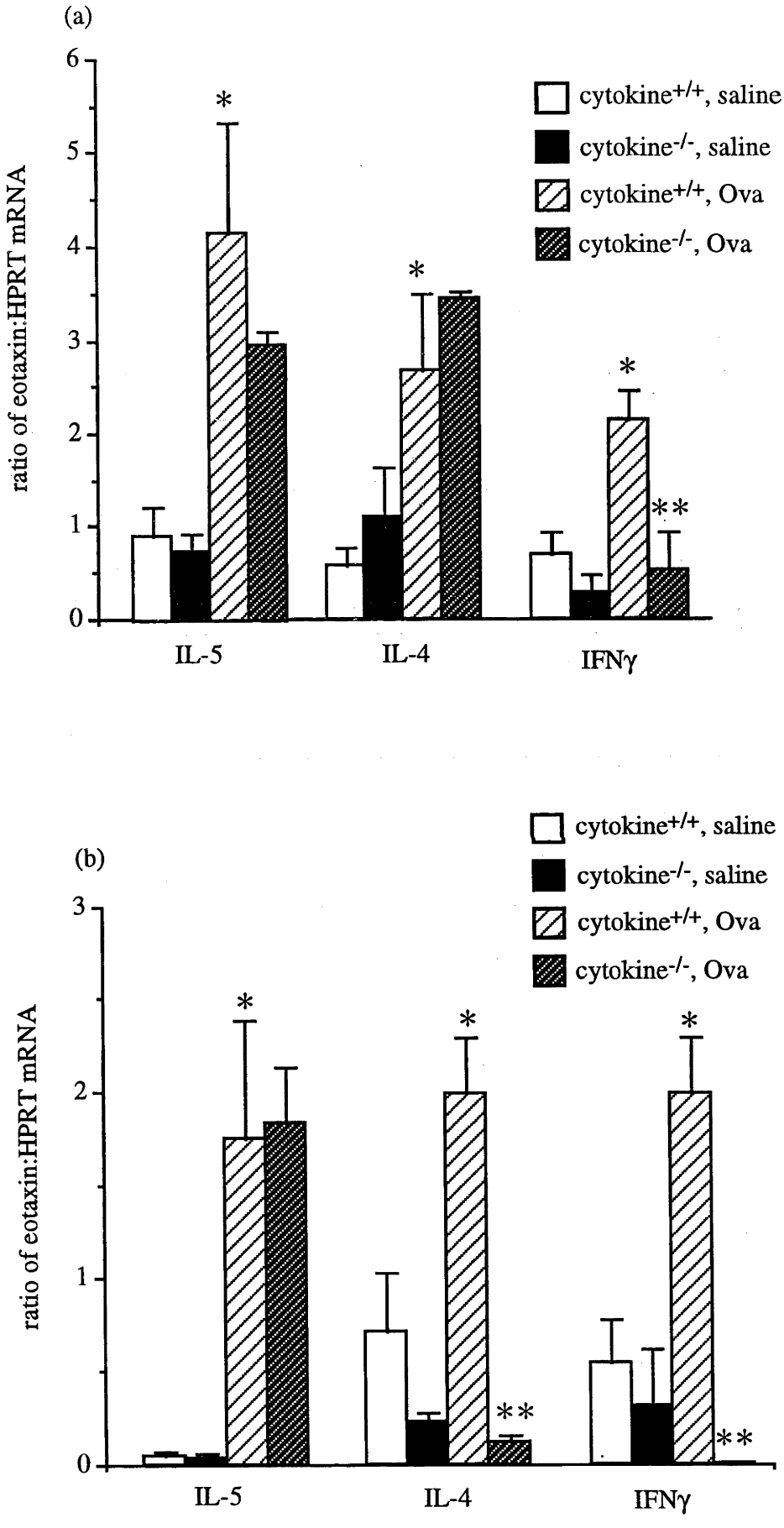

(b)
3
2
1
0
*
*
ratio of eotaxin to HPRT
non-sensitised, saline aerosol
sensitised, Ova aerosol
0
6
12
18
24
hours post aerosol exposure

Figure VI.5 ***The effect of IL-5, IL-4 or IFNγ deficiency on eotaxin expression during allergy of the skin and lung.***

IL-5$^{-/-}$, IL-4$^{-/-}$, IFNγ$^{-/-}$ and the respective wild type mice were sensitised to Ova by i.p. injection on days 0 and 12 and on day 24 cutaneous or airways inflammation was induced in sensitised mice by s.c. Ova administration or Ova aerosol exposure, respectively. The non-sensitised controls received s.c. saline or were exposed to an aerosol of saline instead of Ova exposure. The levels of eotaxin mRNA at skin and lungs sites of allergic inflammation were determined at peak eotaxin expression times [3 hours (in the skin) and 6 hours (in the lung) post antigen provocation]. Eotaxin mRNA expression was compared with HPRT mRNA expression and was determined using semi-quantitative RT-PCR. (a) Significantly higher levels of eotaxin mRNA expression were detectable at skin sites of allergic inflammation in wild type mice when compared with their non-sensitised controls at s.c. sites of saline administration. These elevations in eotaxin expression at sites of cutaneous allergy were significantly reduced in the absence of IFNγ, but not in the absence of IL-5 or IL-4. (b) Significantly higher levels of eotaxin mRNA expression were detectable at sites of allergic airways inflammation in wild type groups when compared with their non-sensitised controls, that were exposed to an aerosol of saline. The elevations in eotaxin expression at sites of allergic airways inflammation were significantly reduced in the absence of either IL-4 or IFNγ, but not in the absence of IL-5. Results represent mean ratio of eotaxin : HPRT mRNA levels of expression ± SEM of groups of 4-8 animals. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test and differences in means were considered significant if $P < 0.05$. (a) $*P < 0.05$ when compared with non-sensitised/saline s.c. injected mice and $**P < 0.05$ when compared with sensitised IFNγ$^{+/+}$ mice administered Ova s.c. (b) $*P < 0.05$ when compared with non-sensitised mice exposed to saline aerosol and $**P < 0.01$ when compared with the sensitised wild type mice exposed to Ova aerosol.


(a)
ratio of eotaxin:HPRT mRNA
6
5
4
3
2
1
0
*
*
*
**
cytokine+/+, saline
cytokine-/-, saline
cytokine+/+, Ova
cytokine-/-, Ova
IL-5
IL-4
IFNγ
(b)
ratio of eotaxin:HPRT mRNA
3
2
1
0
*
*
*
**
**
cytokine+/+, saline
cytokine-/-, saline
cytokine+/+, Ova
cytokine-/-, Ova
IL-5
IL-4
IFNγ

Increased levels of eotaxin mRNA expression were also detected at skin and lung sites of allergic inflammation (at 3 and 6 hours post allergen provocation, respectively) in sensitised IL-$5^{+/+}$ (C57BL6), IL-$4^{+/+}$ (129Sv/C57BL6) and IFN$\gamma^{+/+}$ ($129Sv_{Ev}$) mice (figure VI.5). Furthermore, the elevations were equivalent in all of these groups when compared at the same site and time of analysis (3 hours at skin sites and 6 hours at lung sites).

The increased expression of eotaxin mRNA at skin sites of allergic inflammation was not attenuated in IL-$4^{-/-}$ or IL-$5^{-/-}$ mice (figure VI.5). In contrast, the levels of eotaxin mRNA in the skin did not increase in IFN-$\gamma^{-/-}$ mice following s.c. allergen provocation (figure VI.5). No increase in eotaxin mRNA above basal levels was observed in the lungs of sensitised IFN$\gamma^{-/-}$ mice when measure 6 hours after aeroallergen challenge. However, unlike the preceding experiments in allergic skin, allergen-induced expresion of eotaxin mRNA in the lungs were abolished in sensitised IL-$4^{-/-}$ mice (figure VI.5). No role for IL-5 in the regulation of eotaxin mRNA expression at skin or lung sites of allergic inflammation was demonstrated.

VI.3.3 Eotaxin deficiency alters eosinophilic responses during cutaneous late-phase reaction in mice.

VI.3.3.1 *Eotaxin$^{-/-}$ mice have impaired eosinophilic responses during cutaneous late-phase reaction, but exhibit high responses to s.c. administered saline.*

The accumulation of eosinophils at skin sites of allergic inflammation is biphasic (see section V.3.1.1). When measured at peak eosinophil infiltration times [6 and 24 hours, as determined in C57BL6 (section V.3.1.1) or BALB/c strains of mice (Iwamoto *et al.*, 1992)] significantly greater levels of eosinophils accumulated at sites of Ova-induced CLPR in eotaxin$^{+/+}$ mice in comparison to skin sites of saline administration in non-sensitised controls (figure VI.6). In the absence of eotaxin (as assessed in eotaxin$^{-/-}$ mice) significantly lower level of eosinophils accumulated at sites of CLPR at 6 and 24 hours post s.c. administration of Ova when compared with the eotaxin$^{+/+}$ mice of the same treatment (figure VI.6). Interestingly, significantly higher levels of eosinophils accumulated at skin sites of saline administration in the non-sensitised eotaxin$^{-/-}$ when compared with the wild type mice (figure VI.6). Furthermore, in eotaxin$^{-/-}$ mice, the accumulation of eosinophils at s.c. sites of saline administration in non-sensitised mice were significantly greater than those in sensitised mice at s.c. sites of Ova administration (figure VI.6).

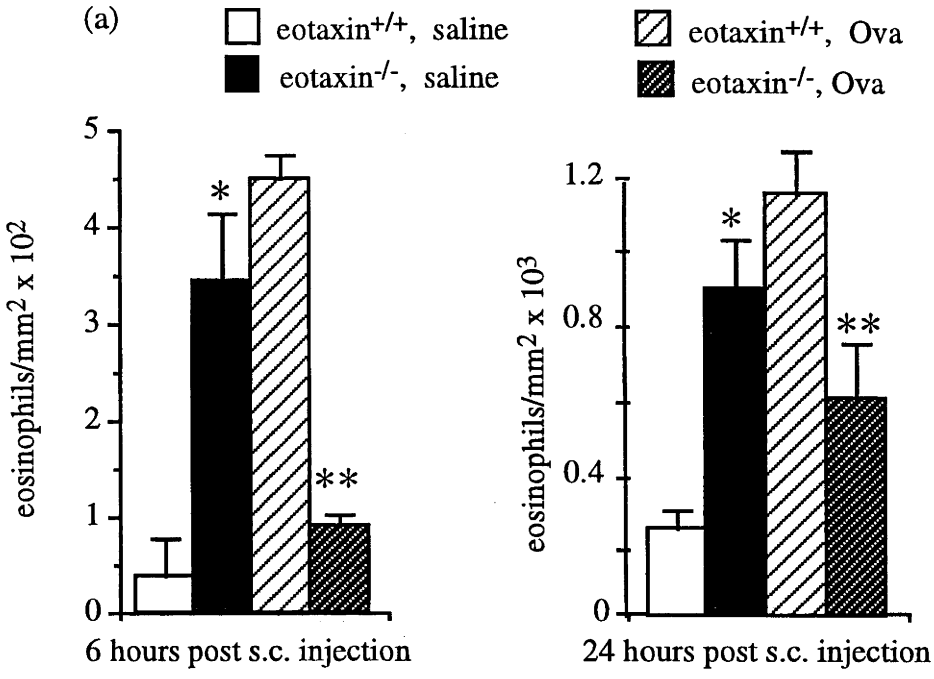


Figure VI.6 ***The role of eotaxin in eosinophil trafficking during CLPR in mice.***

Eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice were sensitised to Ova by i.p injection on days 0 and 12 and on day 24 were challenged with 50 μg Ova by s.c. injection in 100 μl saline. Non-sensitised mice received s.c. saline only. The accumulation of eosinophils at sites of CLPR at 6 (first phase) and 24 (second phase) hours was significantly reduced in the eotaxin$^{-/-}$ mice when compared with the wild types. Significantly greater levels of eosinophils accumulated at s.c. sites of saline administration in the non-sensitised eotaxin$^{-/-}$ mice compared with non-sensitised eotaxin$^{+/+}$ mice at 6 and 24 hours. Furthermore, higher levels of eosinophils accumulated at skin sites of saline administration in the non-sensitised eotaxin$^{-/-}$ mice at 6 hours than at sites of CLPR in the eotaxin$^{-/-}$ mice at the same time. Results represent mean eosinophils/mm^2 ± SEM of groups of 5 animals. The significance of difference between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. *$P < 0.02$ when compared with non-sensitised eotaxin$^{+/+}$ mice given s.c. saline, at the same time point and **$P < 0.02$ when compared with the sensitised eotaxin$^{+/+}$ given s.c. Ova, at the same time point.

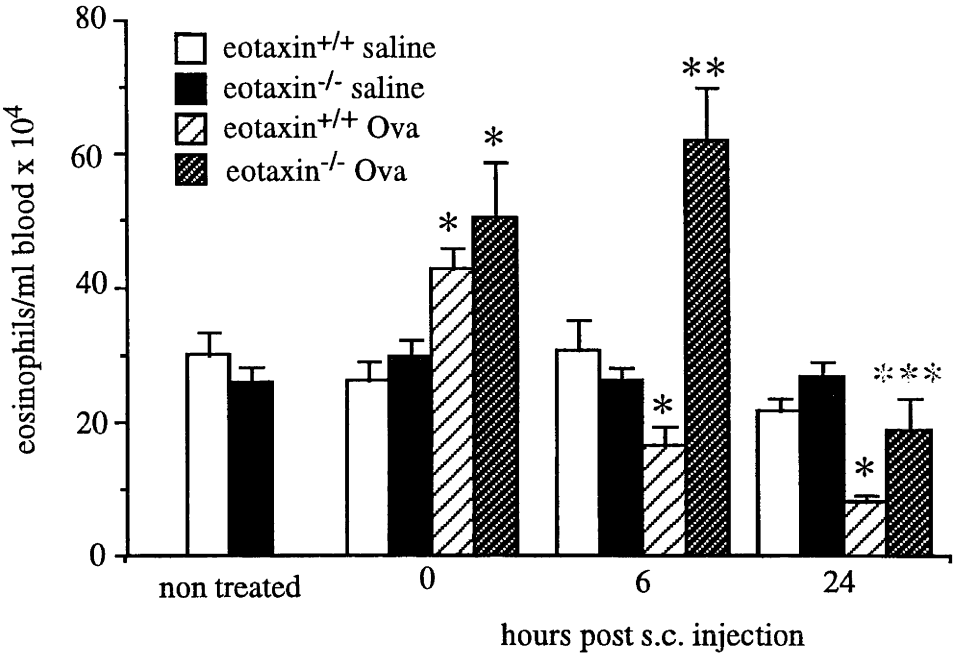


Figure VI.7 ***Levels of circulating eosinophils in eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice during CLPR.***

Eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice were sensitised to Ova by i.p injection on days 0 and 12 and on day 24 were challenged with 50 µg Ova by s.c. injection in 100 µl saline. Non-sensitised mice received s.c. saline only. Sensitised mice displayed higher levels of eosinophils in the blood in comparison to the non-sensitised controls. Following the induction of CLPR, eotaxin$^{+/+}$ mice had significantly lower circulating eosinophil levels in comparison to the non-sensitised saline controls, when compared at the same time point. Circulating levels of eosinophils in these mice at 6 and 24 hours were also significantly lower than the levels in these mice prior to s.c. Ova provocation. In contrast, the sensitised eotaxin$^{-/-}$ mice had significantly higher levels of eosinophils in the blood at 6 hours post Ova administration than the non-sensitised saline eotaxin$^{-/-}$ controls at the same time point. The elevated blood levels of eosinophils in these mice at 6 hours post Ova administration were significantly higher than the levels in these mice prior to the induction of CLPR. There were no differences between the circulating eosinophils levels in the non-sensitised saline controls and the non-treated control group. Results represent mean eosinophils/ml of blood ± SEM of groups of 5 animals. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. *$P < 0.05$ when compared with the non-sensitised eotaxin$^{+/+}$ saline control at the same time point. **$P < 0.002$ when compared with the non-sensitised eotaxin$^{-/-}$ saline controls at 6 hours post s.c. saline injection. There was no significant differenced between the means of the saline injected non-sensitised eotaxin$^{+/+}$ and eotaxin$^{-/-}$ groups when compared at any given time.

*** P<0·05 when compared with sensitized wild type mice at the same time point.

VI.3.3.2 *Eotaxin$^{-/-}$ mice exhibit blood eosinophilia during cutaneous late-phase reaction, in contrast to wild type mice.*

The levels of eosinophils in the circulation of the sensitised eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice were significantly higher than their respective non-sensitised controls (figure VI.7). As previously observed in IL-5$^{+/+}$ mice (see section V.3.1.1), the blood levels of eosinophils in the sensitised eotaxin$^{+/+}$ mice at 6 and 24 hours post s.c. antigen challenge were significantly reduced in comparison to the blood levels of eosinophils of the non-sensitised saline controls or when compared to the blood levels of eosinophils prior to the induction of CLPR (figure VI.7). In contrast, the sensitised eotaxin$^{-/-}$ mice developed a significant blood eosinophilia at 6 hours post s.c. Ova provocation (figure VI.7). At 24 hours post antigen administration these mice no longer exhibited blood eosinophilia compared to the non-sensitised eotaxin$^{-/-}$ saline controls although the level of eosinophils in the blood of the mice were significantly higher than that in the sensitised eotaxin$^{+/+}$ mice (figure VI.7).

VI.3.3.3 *Eotaxin$^{-/-}$ mice have enhanced eosinophilic responses in the bone morrow during cutaneous late-phase reaction compared with wild type mice.*

The levels of eosinophils in the bone marrow of eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice during CLPR were also examined (figure VI.8). The sensitisation of eotaxin$^{+/+}$ mice to Ova induced an increase in the level of eosinophils in the bone marrow in comparison with the non-sensitised and non-treated eotaxin$^{+/+}$ controls (figure VI.8). There was also a reduction in the level of eosinophils in the bone marrow of the sensitised eotaxin$^{+/+}$ mice 24 hours after the s.c. administration of Ova (figure VI.8). In contrast, the s.c. administration of saline to the non-sensitised wild type controls had no effect of the levels of eosinophil in the bone marrow (figure VI.8).

In contrast to wild type mice, the non-sensitised and sensitised eotaxin$^{-/-}$ mice had significantly higher levels of eosinophils in the bone marrow compared to their non-treated groups (figure VI.8). There was a reduction in eosinophil levels in the bone marrow of the sensitised eotaxin$^{-/-}$ mice 6 and 24 hours after the s.c. administration of Ova. Similar to the non-sensitised eotaxin$^{+/+}$ mice there was no change in the levels of eosinophils in the bone marrow of the non-sensitised eotaxin$^{-/-}$ mice following the s.c. administration of saline.

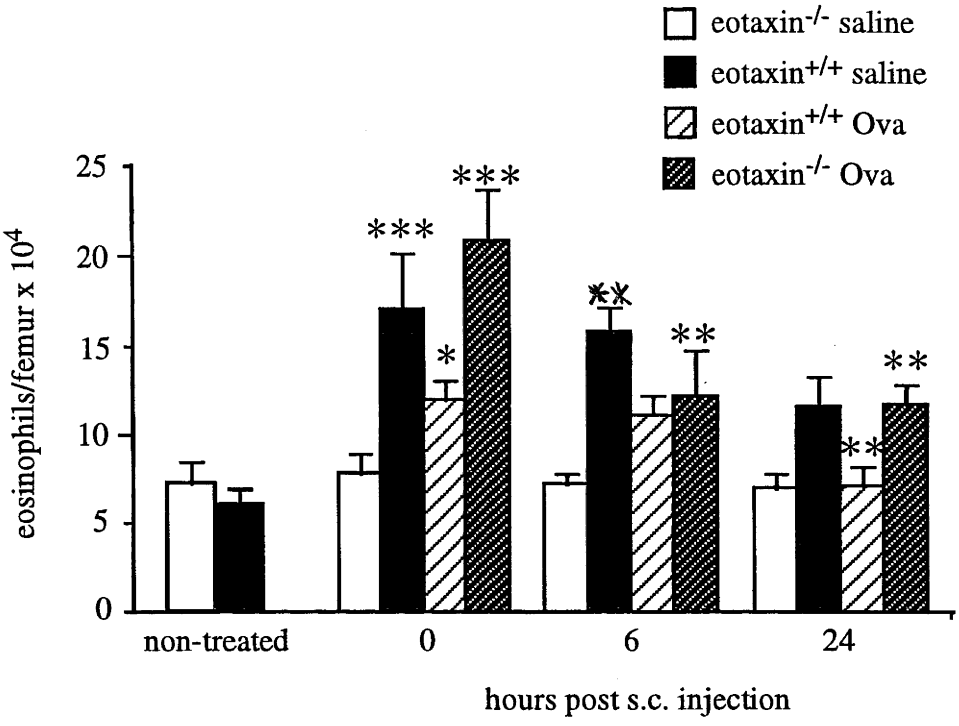


Figure VI.8 ***Levels of eosinophils in the bone marrow of eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice during CLPR.***

Eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice were sensitised to Ova by i.p injection on days 0 and 12 and on day 24 were challenged with 50 µg Ova by s.c. injection in 100 µl saline. Non-sensitised mice received s.c. saline only. Ova sensitisation increased eosinophil levels in bone marrow. Non-sensitised control eotaxin$^{-/-}$ mice also had significantly higher levels of eosinophils in bone marrow compared with non-treated mice. Eosinophil levels in bone marrow were decreased in eotaxin$^{-/-}$ mice at 6 and 24 hours following s.c. Ova provocation. Bone marrow eosinophil levels were also reduced in eotaxin$^{+/+}$ mice following Ova provocation, but only at 24 hours. S.c. administration of saline to non-sensitised eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice did not effect levels of eosinophils in bone marrow. Results represent mean eosinophils/ml of blood ± SEM of groups of 5 animals. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. $*P < 0.05$ when compare with levels in non-treated mice. $**P < 0.01$ when compare with levels prior to s.c. Ova provocation and $***P < 0.02$ when compared with non-treated and non-sensitised groups.

VI.3.4 Eotaxin deficiency does not effect the accumulation of eosinophils in the lungs or the induction of airways hyperreactivity following aeroallergen provocation.

Allergic airways inflammation was induced in eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice to determine if this cytokine has any significant role in the accumulation of eosinophils in the lungs or the induction of airways hyperreactivity, during allergic airways inflammation. The exposure of sensitised eotaxin$^{+/+}$ mice to an aerosol of Ova on days 24 and 26 induced a selective accumulation of eosinophils in the airways on day 27 (figure VI.9). In contrast, the non-sensitised eotaxin$^{+/+}$ mice that were exposed to aerosol of saline for an equivalent duration did not have any significant levels of eosinophils in the BALF.

The sensitised eotaxin$^{-/-}$ mice that were exposed to an aerosol of Ova on days 24 and 26 displayed an equivalent [redacted] and selective (figure VI.9[redacted]) elevation in the levels of eosinophils in the BALF when compared to the wild type mice of the same treatment (figure VI.9). Similar to the observations in eotaxin$^{+/+}$ mice, no significant levels of eosinophil were detectable in the BALF of the non-sensitised eotaxin$^{-/-}$ mice after their exposure to saline aerosol (figure VI.9[redacted]). This was in contrast to observations in the skin where eotaxin deficiency was associated with a hypereosinophilic response at site of saline administration. No significant elevation in the levels of eosinophils in the blood of any of the groups were detected (results not shown).

The duration of aeroallergen exposure in these series of experiments was chosen based upon prior observations that indicated that this was the minimum period of antigen exposure in this model of allergic airways inflammation that was required for the induction of airways hyperreactivity. Associated with the elevation in the levels of eosinophils in the BALF of sensitised eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice (figure VI.9[redacted]) was the development of airways hyperreactivity when compared with the non-sensitised saline aerosol controls of either genotype (figure VI.10). Furthermore, the development of airways hyperreactivity was not impaired or enhanced in the absence of eotaxin.

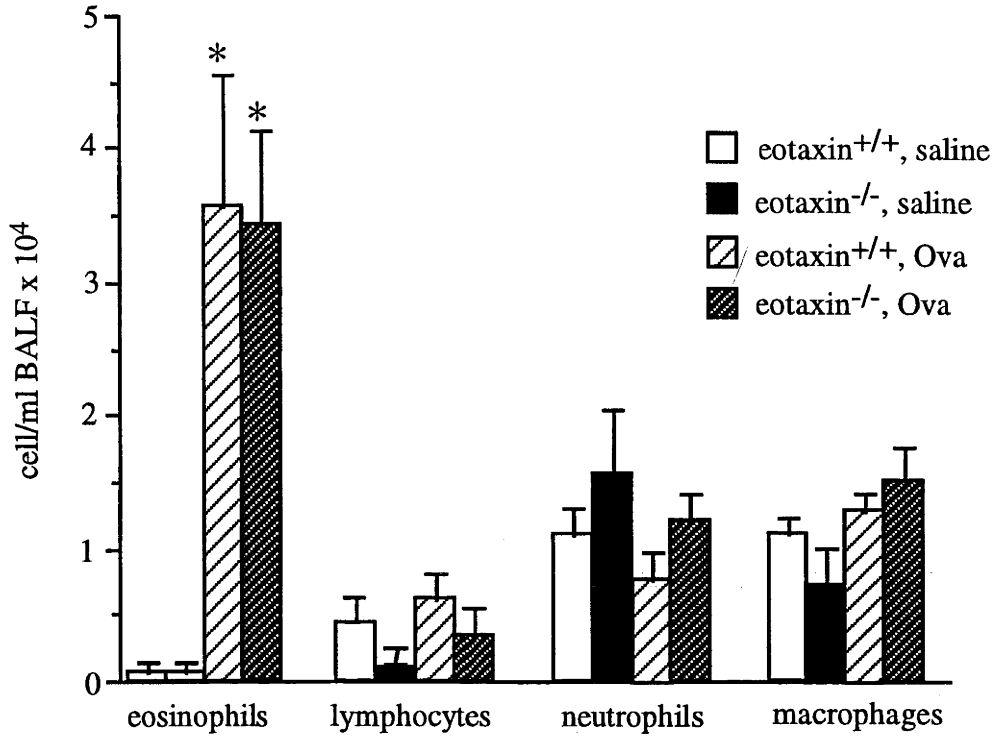


Figure VI.9 ***The levels of leukocytes in BALF of eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice following aeroallergen provocation.***

Eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice were sensitised to Ova by i.p. injection on days 0 and 12 . On days 24 and 26 the sensitised mice were exposed to Ova aerosol and the non-sensitised controls were exposed to saline aerosol only. Leukocytes levels were quantified in the BALF 24 hours after the last aerosol exposure. Ova sensitised/challenged groups had significantly greater levels of eosinophils in BALF when compare with non-sensitised/saline aerosol exposed controls. Levels of lymphocytes, neutrophils and macrophages were not increased in Ova sensitised/challenged groups when compared with non-sensitised/saline aerosol exposed controls. Levels of leukocytes in eotaxin$^{-/-}$ groups were not different from eotaxin$^{+/+}$ groups of the same treatement. Results represent mean cells/ml of BALF ± SEM of groups of 5-6 animals. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. $^*P < 0.001$ when compared with levels in non-sensitised groups.

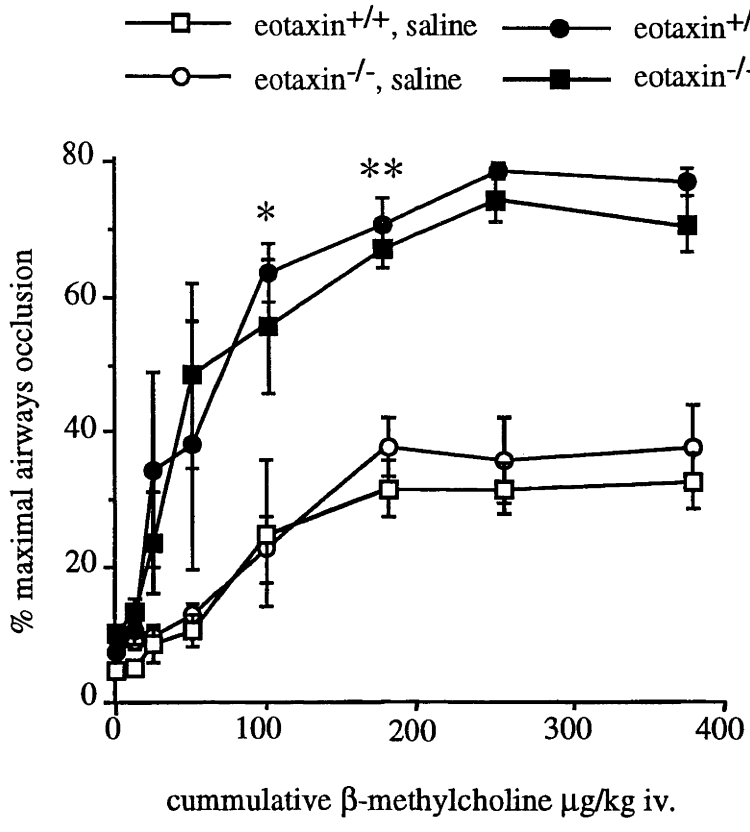


Figure VI.10 ***Eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice exhibit equivalent airways hyperreactivity following aeroallergen provocation.***

Eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice were sensitised to Ova by i.p. injection on days 0 and 12 . On days 24 and 26 the sensitised mice were exposed to Ova aerosol and the non-sensitised controls were exposed to saline aerosol only. Airways hyperreactivity to i.v. β-methylcholine was measured on day 27, 24 hours after the final exposure to aerosol. Ova sensitised/challenged mice exhibited airways hypereactivity when compare with non-sensitised/saline aerosol exposed controls. No significant differences in airways reactivity were detected between eotaxin$^{+/+}$ and eotaxin$^{-/-}$ groups of the same treatment. Airways reactivity was measured by monitoring i.v. β-methylcholine-induced changes in respiratory overflow volume. Data represents mean percentage airway occlusion ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. $^{*}P < 0.05$ and $^{**}P < 0.01$ when compare with saline controls at the same dose of β-methylcholine.

VI.4 DISCUSSION.

Mice constitutively express eotaxin in numerous tissues and it has been suggested that this chemokine may regulate the basal migration of eosinophils through tissues (Rothenberg *et al.*, 1995b). Eotaxin$^{-/-}$ mice also have lower levels of eosinophils residing in the intestinal mucosa and in the thymus (levels have not been characterised in other tissues) (Mathews *et al.*, 1998). In addition, eotaxin was thought to regulate basal levels of circulating eosinophils (Rothenberg *et al.*, 1997), although the eotaxin$^{-/-}$ mice used in this study did not have lower circulating levels of eosinophils when compared with eotaxin$^{+/+}$ mice. Interestingly, the i.v. administration of eotaxin to eotaxin$^{-/-}$ mice induced a blood eosinophilia that was significantly greater than that induced by this chemokine in wild type mice. Furthermore, the movement of eosinophils into the circulation of both eotaxin$^{-/-}$ and eotaxin$^{+/+}$ mice in response to i.v. eotaxin did not require the mobilisation of bone marrow stores of this leukocyte. These results suggest that the enhanced blood eosinophilia in the eotaxin$^{-/-}$ mice in response to the i.v. eotaxin, involved a greater sequestrating of eosinophils out of tissues into the circulation. An explanation for the hypereosinophilic response to eotaxin in eotaxin$^{-/-}$ mice may involve sensitisation of signalling through the CCR3 receptor. C-C chemokines induce transient Ca^{2+} fluxes in eosinophils which are coupled with chemotactic responses and regulation of adhesion molecule expression (Forssmann *et al.*, 1997). The exposure of eosinophils to eotaxin has been shown to desensitise these cells to subsequent Ca^{2+} responses elicited by eotaxin or other CCR3 binding CC-chemokines (Forssmann *et al.*, 1997). In wild type mice, basal levels of eotaxin may modulate CCR3 signalling pathways that constitutively regulate chemotactic responsiveness of eosinophils. In the absence of such regulation, eosinophils may become hyperresponsive to chemokines that signal thorough CCR3. Thus, eotaxin may regulate eosinophil function by directly modulating CCR3 function and subsequent migratory processes. Interestingly, the action of IL-5 on the induction of blood eosinophilia occurs independently of eotaxin, supporting the concept that these two cytokines may act via different mechanisms to induce blood eosinophilia in mice (Chapter IV).

In eotaxin$^{-/-}$ mice, IL-5-induced blood eosinophilia correlated with the mobilisation of eosinophils from the bone marrow and this response was not diminished in the absence of eotaxin. These results suggest that eotaxin is not required to mobilise this pool of eosinophils. However, the rapid induction of eosinophilia in the bone marrow of eotaxin$^{-/-}$ mice following the i.v. administration of IL-5 suggests that eotaxin may play a role in the regulation of IL-5-induced eosinophil differentiation. Notably, eosinophil progenitor cells from eotaxin$^{-/-}$ mice were hyperresponsiveness to IL-5 *in vitro* (figure VI.3). This suggests that eotaxin plays a regulatory role with IL-5 in eosinophil differentiation in the bone marrow. However, eotaxin$^{-/-}$ mice have normal resting levels

of eosinophils in the bone marrow (compared to wild types mice), suggesting that eotaxin does not have an obligatory role in basal eosinophil differentiation.

In an attempt to determine what cytokines regulate eotaxin expression, at baseline and during allergy, levels of mRNA expression were characterised in factor deficient mice. Constitutive eotaxin mRNA expression was observed in the lungs and in the skin. Interestingly, other investigations have failed to detect mRNA for this chemokine in either of these tissues in mice (Gonzalo *et al.,* 1996). Notably, eotaxin expression was upregulated at sites of allergic inflammation and increased expression occurred soon after antigen exposure, supporting a role for this chemokine in early eosinophil trafficking events (Rothenberg *et al.*, 1997). The prolonged expression of eotaxin in the allergic lung compared to allergic skin suggests a differential temporal role for eotaxin in the regulation of eosinophil trafficking at different tissue sites of allergic disease. Notably, the elevations in expression of eotaxin mRNA at both sites of allergic inflammation were dependent on IFN-γ and independent of IL-5. Interestingly, antigen-induced increases in eotaxin expression in the lung, but not in the skin, were also dependent on IL-4. Thus, IL-4 may selectively regulate the expression of eotaxin in the lungs. These results suggest that eotaxin expression may be differentially regulated by cytokines at mucosal (lung) and non-mucosal sites (skin) of allergic inflammation. The observation that the expression of eotaxin mRNA in allergic lungs did not increase in the absence of either IL-4 or IFNγ, suggests that these molecules may act together to activate eotaxin expression.

Although the expression of eotaxin mRNA was upregulated in the skin and in the lungs at sites of antigen provocation, eosinophil recruitment at sites of allergic inflammation was dependent on eotaxin only in the skin. Elevated expression of eotaxin mRNA at sites of CLPR was observed in wild type mice at 3 hours post antigen administration, but not at later time points. Interestingly, the accumulation of eosinophils at sites of CLPR was impaired during both the early (6 hours) and late (24 hours) infiltration phases in eotaxin$^{-/-}$ mice. This suggest that either eotaxin is very stable and remains active at sites of cutaneous inflammation for long periods or that eotaxin activates a second pathway leading to the generation of other eosinophil chemoattractants. Furthermore, levels of circulating eosinophils at 6 and 24 hours post s.c. antigen administration in eotaxin$^{-/-}$ mice were not diminished in contrast to wild type mice indicating that eosinophil recruitment into sites of CLPR was impaired. In addition, there was a direct correlation between the decreased accumulation of eosinophils at sites of CLPR and an elevation in the levels of eosinophils in the circulation. Interestingly, there was a decrease in the levels of eosinophils in the bone marrow of eotaxin$^{+/+}$ mice during the later stages, but not early stages of CLPR (figure VI.8). These results suggest that eosinophil trafficking during the early stages of CLPR does not solely rely on a pronounced mobilisation of

eosinophils from the bone marrow, and thus may depend on the available circulating pool of eosinophils. In contrast, the levels of eosinophils in the bone marrow of eotaxin$^{-/-}$ mice were reduced during the early stages of CLPR as well as during the later stages. Early mobilisation of eosinophils from the bone marrow may account for the increased blood levels of eosinophils in the eotaxin$^{-/-}$ mice during the early phase of CLPR. These observations may reflect the hypersensitivity of eosinophil progenitor differentiation to IL-5 in eotaxin$^{-/-}$ mice that was observed *in vitro* (figure VI.3). However, Although additional inflammatory factors may act with IL-5 to facilitate eosinophil mobilisation from bone marrow during allergy since i.v. administration of IL-5, in isolation, enhanced eosinophil levels in the bone marrow, but not the blood, of these animals. These results suggests that eosinophil mobilisation from the bone marrow during allergy is regulated, in part, by eotaxin.

The accumulation of eosinophils at sites of CLPR was reduced in eotaxin$^{-/-}$ mice. Furthermore, blood eosinophil levels did not decrease in eotaxin$^{-/-}$ mice following antigen provocation (in contrast to wild type mice) indicating that eotaxin$^{-/-}$ mice had impaired eosinophil recruitment. Collectively, these data show a role for eotaxin in eosinophil trafficking during CLPR. Interestingly, non-sensitised eotaxin$^{-/-}$ mice accumulated high levels of eosinophils at skin sites of saline administration and these results suggest that eotaxin negatively regulates eosinophil trafficking in the skin in response to non-specific stimuli (such as tissue trauma). This is despite the observation that following s.c. administration of saline, eosinophil levels in the circulation of eotaxin$^{-/-}$ mice were not significantly different from those of wild type mice. Furthermore, the pronounced eosinophilic response at s.c. sites of saline administration in eotaxin$^{-/-}$ mice was not associated with any decrease in the levels of eosinophils in the blood or bone marrow, unlike responses to s.c. Ova in sensitised eotaxin$^{-/-}$ mice. The accumulation of eosinophils at sites of saline administration may involve the production of IL-5, LTB_4 or IL-8 (Oliveira *et al.*, 1996) and hypereosinophilic responses in eotaxin$^{-/-}$ mice may be due to increased sensitivity of eosinophils to these molecules. Although the mechanism underlying enhanced response to saline in eotaxin$^{-/-}$ mice remains to be elucidated, these observations further support a role for eotaxin in the regulation of eosinophil trafficking at base line and perhaps in response to tissue injury.

Unlike wild type mice, the i.p. injection of eotaxin$^{-/-}$ mice with adjuvant (Alhydrogel) in saline (which serves as a control for the Ova i.p. sensitisation) induced an elevation in the level of eosinophils in the bone marrow. Intraperitoneal administration of saline to rats has been shown to induce a peritoneal eosinophilia which may be regulated by the local production of eosinophil chemoattractants, including IL-5 (Oliveira *et al.*, 1996). The production of IL-5 in response to saline administration may induce eosinophil

differentiation in the bone marrow of eotaxin$^{-/-}$ mice due to the haemopoietic sensitivity to this cytokine in these animals.

Interestingly, in contrast to the trafficking of eosinophils at sites of CLPR, no role for eotaxin in the accumulation of eosinophils to the allergic lung was observed. Previous experiments by Rothenberg *et al.*, (1997) also failed to find differences between the number of eosinophils recruited to the airways of eotaxin$^{-/-}$ and eotaxin$^{+/+}$ mice during late-phase responses. However, the accumulation of eosinophils in the lungs during the early stage of allergic airways inflammation was significantly impaired in the absence of eotaxin (Rothenberg *et al.*, 1997). Previously it was shown (Chapter V) that the development of airways hyperreactivity in both IL-5$^{+/+}$ and IL-5$^{-/-}$ mice correlates with the levels of eosinophils and MBP in the BALF. This suggest that in this model of allergic airways inflammation, the development of airways hyperreactivity is mediated by eosinophils and is associated with the presence of MBP. Since allergic eotaxin$^{+/+}$ and eotaxin$^{-/-}$ mice exhibited equivalent elevations in airways eosinophilia and responsiveness to β-methylcholine (above the non-allergic controls), it was concluded that eotaxin has no obligatory role in eosinophil activation/degranulation and the induction of airways hyperreactivity during the late-phase reaction.

In conclusion, eotaxin regulates eosinophilic responses at basal states in the blood and in tissues (in response to non-specific stimuli). This chemokine also modulates the effect of IL-5 on haeomopoiesis. During allergic responses, eotaxin is produced at sites of inflammation and the stimulation of expression is differentially controlled by the cytokines IFNγ and IL-4 in the skin (non-mucosal) and the lung (mucosal). Eotaxin promotes eosinophil recruitment to sites of CLPR, but is not required for the recruitment of eosinophils into the lungs, nor for the development of airways hyperreactivity, during the later stages of allergic inflammation. Although these results indicate that eotaxin regulates eosinophil function, this chemokine does not play an obligatory role in this process.

- where other eosinophil chemoattractants such as IL-5 and RANTES may have a greater role.

CHAPTER VII

THE ROLE OF IL-5, IL-4, TNF-α and IL-1β IN EOSINOPHIL RECRUITMENT DURING ALLERGIC CUTANEOUS LATE-PHASE REACTIONS IN MICE

VII.1 INTRODUCTION.

Elevated levels of IL-5 are found in the BALF and sera of asthmatics and at sites of allergic inflammation in mice (Walker *et al.*, 1992; Ohkawara *et al.*, 1997). IL-5 has also been shown to play a key role in regulating blood eosinophilia and thus, subsequent tissue eosinophilia, during allergic responses in mice (Foster *et al.*, 1996) demonstrating the importance of this molecule in eosinophil trafficking. Furthermore, eosinophil recruitment to sites of chemotactic stimuli (IL-5 and eotaxin, Chapter IV) and at base line is markedly attenuated in the absence of IL-5 suggesting that this cytokine may also play important roles in modulating the accumulation of eosinophils in tissues by activating homing and adhesion pathways.

However, although IL-5 may regulate eosinophil trafficking at various steps in the recruitment mechanism during allergy and during baseline, this cytokine does not play an obligatory role in eosinophil homing during allergic CLPR (section V.3.1).

A number of cytokines, through activation of adhesion molecules, may also play key roles in regulating eosinophil migration into tissues during allergic responses. In particular, IL-4, TNF-α and IL-1β have been implicated *in vitro* in the regulation of eosinophil transmigration by upregulating ICAM-1 and VCAM-1 expression on the vascular endothelium (Lamas *et al.*, 1988; Schleimer *et al.*, 1992; Ebisawa *et al.*, 1992; Briscoe *et al.*, 1992; Shimizu *et al.*, 1992; Wardlaw, 1993; Acevedo *et al.*, 1993; Pober *et al.*, 1996). Eosinophil recruitment to sites of allergic inflammation in the lung is dependent on ICAM-1 and VCAM-1 in mice (Gonzalo *et al.*, 1996a). Furthermore, elevated levels of IL-4 and TNF-α are observed in the sera and BALF of sensitised mice following allergen provocation (Ohkawara *et al.*, 1997) and this implies that these molecules activate adhesion systems during allergic responses.

Although IL-4, TNF-α and IL-1β upregulate adhesion pathways used by eosinophils and are found at sites of allergic inflammation, their combined and individual roles in regulating eosinophil migration to sites of allergen provocation are unknown. Recently, a role for IL-4, TNF-α and IL-1β individually (but not IL-5), in the promotion of eosinophil recruitment to sites of hapten administration in a murine model of cyclophosphamide-induced tissue eosinophilia was demonstrated (Satoh *et al.*, 1997). These studies suggest that neither IL-4, TNF-α nor IL-1β are redundant in the trafficking of eosinophils, since mAbs to each of these cytokines inhibited eosinophil recruitment at sites of hapten administration. In contrast, anti-IL-5 mAb had no effect on the accumulation of eosinophils, suggesting that local IL-5 is not required for eosinophil homing or that this cytokine is not involved in hapten-induced eosinophilia. Humoral responses are strongly inhibited by cyclophosphamide and the adhesion pathways utilised

during hapten-induced eosinophilia may differ from those required for allergic eosinophilia.

The aim of experiments in this chapter was to define the role that IL-4, TNF-α and IL-1β play in regulating tissue eosinophilia during CLPR, with a view to identifying the elemental signals regulating eosinophil homing during allergic responses. The relationship between IL-5 and these cytokines for eosinophil recruitment to sites of CLPR was also determined. In addition, since IL-5, IL-4, TNF-α and IL-1β have all been implicated in activating ICAM-1 and VCAM-1 adhesion systems, the usage of these two adhesion pathways by eosinophils during recruitment processes to sites of allergic CLPR was also determined.

VII.2 MATERIALS AND METHODS.

VII.2.1 Dissecting the role of adhesion systems involved in eosinophil recruitment during cutaneous late-phase reaction.

CLPR was induced in mice (C57BL6, male, 6-8 weeks of age) (see section V.2.1.1). On day 24, the mice received a i.v. co-injection of 100 μg of rat anti-mouse ICAM-1 (clone; YN1, a gift from Dr N. King, University of Sydney, Australia), and rat anti-mouse VCAM-1(clone; MK1.9, a gift from Dr M. E. Rothenberg, Childrens Hospital Medical Centre, Cincinnati, OH., USA.) or 100 μg of either of these anti-adhesion molecule antibodies plus 100 μg of the control IgG mAb (clone; GL113) to keep the total amount of antibody given constant. The control group received 200 μg of control rat IgG (GL113). Thirty minutes later, CLPR was induced by s.c. injection of 50 μg of Ova in 100 μl of 0.9% saline. The levels of eosinophils at skin sites of Ova administration and in the circulation of the mice were quantified 6 hours after the s.c. administration of Ova (see sections II.2.4 and II.2.2). The accumulation of eosinophils was measured at 6 hours (the peak of the first phase of eosinophil infiltration into the skin) rather that at 24 hours (the peak of the second phase of eosinophil recruitment) because eosinophil recruitment was reported to be partially independent of IL-5 at 6 hours and dependent on IL-5 at 24 hours (Iwamoto *et al.*, 1994).

VII.2.2 Analysing the role of TNF-α, IL-4, and IL-1β in the recruitment of eosinophils at sites of cutaneous late-phase reaction in IL-5$^{-/-}$ mice.

IL-5$^{-/-}$ mice were sensitised to Ova by i.p. injection on days 0 and 12 (see section V.2.1.1). On day 24, the mice were given a s.c. administration of 0.9 mls of air and 50 μg of Ova in 100 μl of saline containing 20 μg of rat anti-mouse TNF-α mAb (clone; G281-2626; Pharmingen, San Diago, CA., USA.) and/or 20 μg of rat anti-mouse IL-4 mAb (11B11) and/or 20 μg of hamster anti-mouse IL-1β mAb (Genzyme, Cambridge, MA., USA.) or 60 μg of isotype control antibodies [40 μg of rat IgG, clone; GL113 and 20 μg of hamster anti-TNP IgG (clone; G235-2356, Genzyme, Cambridge, MA., USA.)]. The total amount of antibody given to any one animal was maintained at 60 μg by substituting isotype control mAbs for the respective monoclonal rat anti-mouse TNF-α (rat IgG), rat anti-mouse IL-4 (rat IgG) or hamster anti-mouse IL-1β antibodies (hamster anti-TNP IgG). The accumulation of eosinophils at skin sites of Ova administration and eosinophil levels in the circulation were quantified at 6 hours post s.c. administration of Ova (see sections II.2.4 and II.2.2). Eosinophilic responses in the skin and in the blood were also analysed at 6 hours rather than at 24 hours post Ova administration as describe above, but also so that comparisons could be made between

these cytokines and ICAM-1- and VCAM-1- dependent eosinophil recruitment pathways (as outlined in section VII.2.1.)

VII.2.3 Analysis of the role of TNF-α and IL-4 in eosinophil recruitment mechanisms during cutaneous late-phase reaction in wild type mice.

Since the treatment of IL-5$^{-/-}$ mice with anti-TNF-α or anti-IL-4 mAs reduced the accumulation of eosinophils in the skin at sites of Ova provocation, the role of these cytokines in eosinophil recruitment in wild type mice (ie. in the presence of IL-5) at sites of CLPR was also examined. CLPR was also induced (see section V.2.1.1) in TNF receptor deficient (P55/P75$^{-/-}$) mice, IL-4$^{-/-}$ mice and their wild type littermates (both 129Sv/C57BL6 cross). - A gift from Allistar Ramsay (CICSMR) P55/P75$^{-/-}$ and IL-4$^{-/-}$ mice were used preferentially in these experiments instead of treating wild type mice with antibodies against TNF-α and IL-4. The accumulation of eosinophils at skin sites of Ova administration and eosinophil levels in the circulation were quantified at 6 hours post s.c. administration of Ova on day 24 (see sections II.2.4 and II.2.2). Non-sensitised control received a s.c. injection of saline and the accumulation of eosinophils at injection sites and in the circulation were also quantified 6 hours later (see sections II.2.4 and II.2.2).

Since a reduction in the accumulation was observed at sites of CLPR in the IL-4$^{-/-}$ mice, eosinophil homing to sites of CLPR was examined at 6 hours post s.c. injection on day 24, by monitoring the homing of adoptively transferred H33342-labelled eosinophils to sites of Ova or saline administration (see section V.2.1.3).

Because anti-IL-4 and anti-TNF-α mAbs each reduced the accumulation of eosinophils at sites of CLPR in IL-5$^{-/-}$ mice (see section VII.3.2), but not in wild types (see section VII.3.3), IL-4 and TNF-α may have overlapping functions with IL-5 in the promotion of eosinophil recruitment at sites of allergic inflammation. To determine if IL-5 could take the place of IL-4 and TNF-α to promote eosinophil recruitment at sites of allergic inflammation, eosinophil recruitment 6 hours after Ova provocation (CLPR, see section IV.2.2.1) was assessed in wild type mice treated with 20 μg of rat anti-mouse TNF-α mAb (Pharmingen, San Diago, CA., USA., clone; G281-2626) and 20 μg of rat anti-mouse IL-4 (clone; 11B11). The control group received 40 μg of isotype control mAb (clone; GL113) and all of these mAbs were co-administered s.c. with the 50 μg of Ova. Antibody treatments were used in this experiment since mice deficient for both TNF-α and IL-4 were unavailable.

VII.3 RESULTS.

VII.3.1 Eosinophils utilise both ICAM-1 and VCAM-1 adhesion pathways during recruitment at sites of cutaneous late-phase reaction.

Pretreatment of mice with anti-ICAM-1 or anti-VCAM-1 mAbs caused a significant reduction in the accumulation of eosinophils at sites of Ova-induced CLPR, when measured at 6 hours (figure VII.1a). Furthermore, the simultaneous treatment of mice with both of these mAbs was significantly more effective at inhibiting the accumulation of eosinophils at sites of allergic inflammation in the skin than either one alone (figure VII.1a). Treatment of mice with the control antibody had no effect on eosinophilic responses in the skin of mice when compared to groups that did not receive antibody (figure VII.1a)

The levels of eosinophils in the blood of mice that received anti-VCAM-1, anti-ICAM-1 or control mAbs i.v. were inversely proportional to level of accumulation of this cell at the site of CLPR, when measured at 6 hours. The mice that received anti-ICAM-1 and/or anti-VCAM-1 mAbs had significantly ($P < 0.05$) higher levels of eosinophils in the blood 6 hours after the s.c. administration of Ova when compared with the control mAb treated mice. Eosinophilic responses in the blood of mice at 6 hours were not affected by control antibody treatment (figure VII.1b).

VII.3.2 IL-4 and TNF-α have important roles in eosinophil recruitment during cutaneous late-phase reaction.

The roles of IL-4, TNF-α and IL-1β in the recruitment of eosinophils at sites of CLPR were examined in IL-5$^{-/-}$ mice. The s.c. treatment of IL-5$^{-/-}$ mice with control mAb did not effect the accumulation of eosinophils at sites of CLPR at 6 hours (figure VII.2a). Treatment with anti-IL-4 or anti-TNF-α mAbs significantly inhibited the accumulation of eosinophils at sites of Ova-induced CLPR at 6 hours. Interestingly, the effect of both of these mAbs together was not greater than the effect of either one of these mAbs alone. The treatment of mice with anti-IL-1β mAb had no effect on the accumulation of eosinophils.

The levels of eosinophils in the blood of sensitised wild type and IL-5$^{-/-}$ mice were significantly reduced following s.c. Ova provocation in the absence of antibody treatment (see section V.3.1). The s.c. administration of the control mAbs at sites of Ova provocation did not affect eosinophilic responses in the blood at 6 hours in IL-5$^{-/-}$ mice (figure VII.2b).

Figure VII.1 ***Eosinophil recruitment at sites of cutaneous late-phase reaction in IL-5$^{+/+}$ and IL-5$^{-/-}$ mice is dependent on both ICAM-1 and VCAM-1 adhesion pathways*** .

IL-5$^{+/+}$ and IL-5$^{-/-}$ mice were sensitised to Ova by i.p. injection on days 0 and 12. On day 24 the mice received an i.v. injection of (1) rat anti-mouse ICAM-1 (100 μg) and rat anti-mouse VCAM-1 (100 μg); isotype control mAb (GL113, 200 μg); rat anti-mouse ICAM-1 and isotype control mAb (GL113, 100 μg) or rat anti-mouse VCAM-1 (100 μg) and isotype control mAb (GL113, 100 μg). After administration of antibodies a s.c. co-injection of 50 μg of Ova in 100 μl of saline plus 900 μl of air was performed. To compare the effect of control antibody treatment on eosinophilic responses at s.c. injection sites and in the blood sensitised mice were given a s.c. injection Ova and air and non-sensitised mice were given a s.c injection of saline and air only in the absence of i.v. mAb. All groups were sacrificed at 6 hours post s.c. injections and eosinophils at skin sites and in the circulation quantified. (a) The treatment of IL-5$^{+/+}$ and IL-5$^{-/-}$ mice with anti-ICAM-1 or anti-VCAM-1 mAbs reduced the accumulation of eosinophils at sites of CLPR. Furthermore, in combination these antibodies further inhibited the accumulation of eosinophils at skin sites. Data represents mean eosinophils/mm^2 ± SEM of groups of 5 mice. (b) As above for (a) the Ova-induced reduction in the levels of eosinophils in the circulation at 6 hours. Data represents the mean eosinophils/ml blood ± SEM of groups of 5 animals. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $p < 0.05$. (a) *$P < 0.05$ when compared with the mice that were treated with the isotype control mAb and ** $p < 0.05$ compared with the mice that received anti-ICAM-1 or anti-VCAM-1 mAbs. (b) *$P < 0.05$ when compared with eosinophil levels before s.c. administration of Ova.

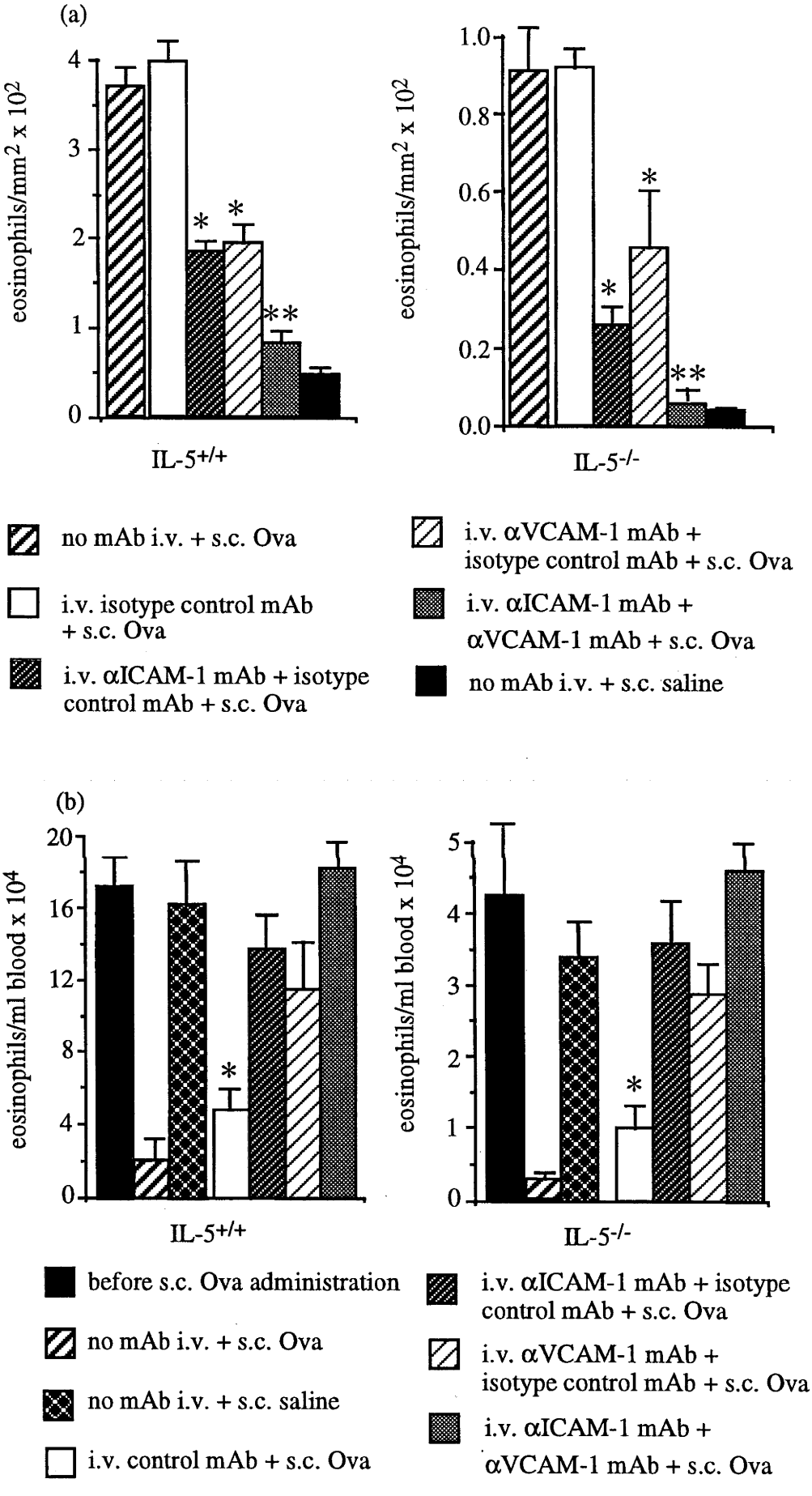
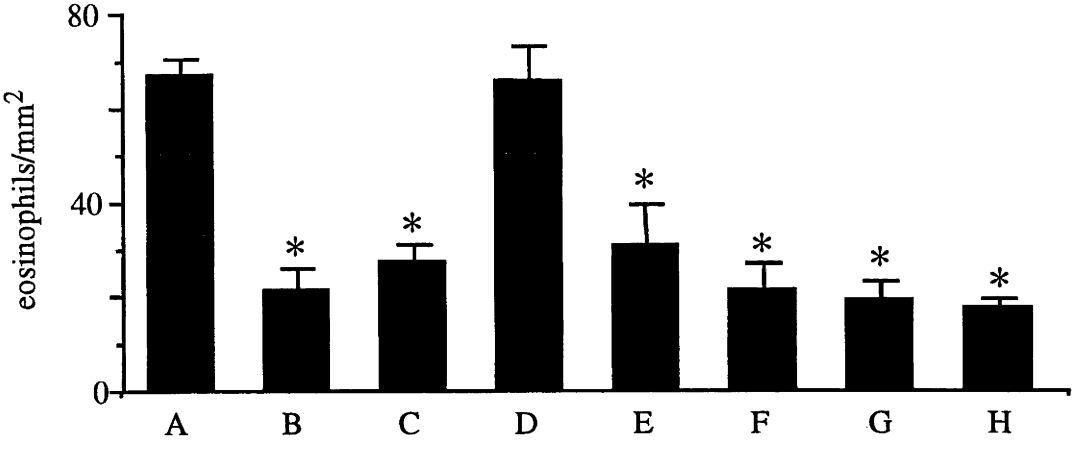

(a)
eosinophils/mm^2 x 10^2
IL-5$^{+/+}$
IL-5$^{-/-}$
no mAb i.v. + s.c. Ova
i.v. isotype control mAb + s.c. Ova
i.v. αICAM-1 mAb + isotype control mAb + s.c. Ova
i.v. αVCAM-1 mAb + isotype control mAb + s.c. Ova
i.v. αICAM-1 mAb + αVCAM-1 mAb + s.c. Ova
no mAb i.v. + s.c. saline
(b)
eosinophils/ml blood x 10^4
IL-5$^{+/+}$
IL-5$^{-/-}$
before s.c. Ova administration
no mAb i.v. + s.c. Ova
no mAb i.v. + s.c. saline
i.v. control mAb + s.c. Ova
i.v. αICAM-1 mAb + isotype control mAb + s.c. Ova
i.v. αVCAM-1 mAb + isotype control mAb + s.c. Ova
i.v. αICAM-1 mAb + αVCAM-1 mAb + s.c. Ova

Figure VII.2 ***Eosinophil recruitment during cutaneous late-phase reaction is inhibited in IL-5$^{-/-}$mice in the presence of anti-TNF-α or anti-IL-4 mAbs.***

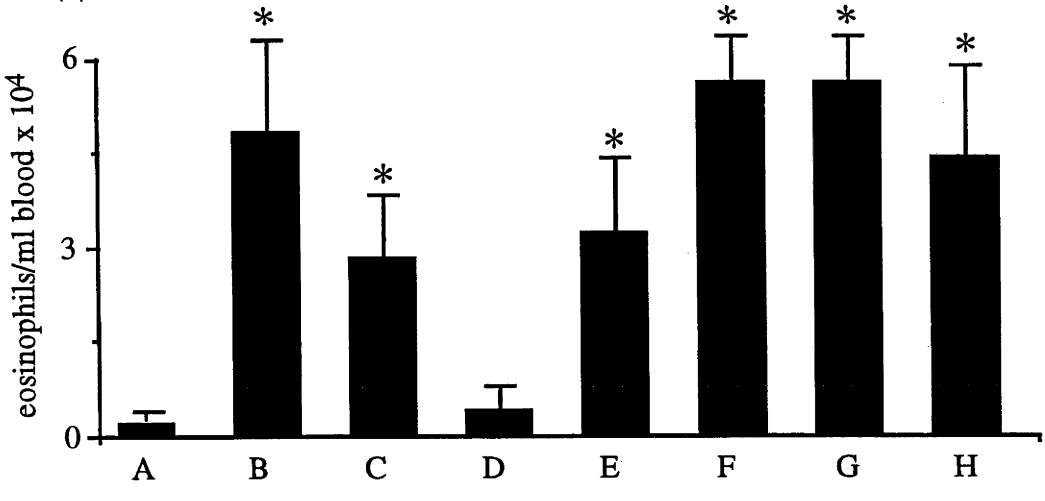
IL-5$^{-/-}$ mice were sensitised to Ova by i.p injection on days 0 and 12. On day 24 mice received a s.c. injection containing 50 μg Ova and combinations of 20 μg of anti-IL-4 (or isotype control) mAb, 20 μg of anti-TNF-α (or isotype control) mAb and 20 μg of anti-IL-1β (or isotype control) mAb in saline. The accumulation of eosinophils at skin sites and eosinophil levels in the blood were determined 6 hours post s.c. injection with Ova. (a) Treatment of mice with anti-IL-4 or anti-TNF-α mAb reduced the accumulation of eosinophils in the skin. In contrast, anti-IL-1β mAb treatment did not effect the accumulation of eosinophils. The coadministration of IL-4 and TNF-α did not further reduce the accumulation of eosinophils at sites of CLPR when compared with responses induced by treatment with either of mAbs alone. Data represents mean eosinophils/mm^2 ± SEM of groups of 5 animals. (b) Blood levels of eosinophils in mice treated with anti-IL-4 mAb and/or anti-TNF-α mAb and/or anti IL-1β mAb were inversely proportional to the levels of eosinophils that accumulated at skin sites of CLPR. The IL-5$^{-/-}$ mice that received anti-IL-4 and/or anti-TNF-α mAbs had significantly higher levels of eosinophils in the blood compared with control antibody treated mice. Anti-IL-1β mAb, but not anti-IL-4 or anti-TNF-α mAb treatments displayed similar eosinophil levels in the blood when compared with control antibody treated mice. Data represents mean eosinophils/ml blood ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. $^*P < 0.05$ compared with the isotype control mAb treated mice.

(a)



A - Isotype control mAbs
B - anti-IL-4 mAb
C - anti-TNFα mAb
D - anti-IL-1β mAb
E - anti-TNFα + anti-IL-1β mAb
F - anti-IL-4 + anti-IL-1β mAb
G - anti-IL-4 + anti-TNFα mAb
H - anti-IL-4 + anti-TNFα + anti-IL-1β mAb

(b)



A - Isotype control mAbs
B - anti-IL-4 mAb
C - anti-TNFα mAb
D - anti-IL-1β mAb
E - anti-TNFα + anti-IL-1β mAb
F - anti-IL-4 + anti-IL-1β mAb
G - anti-IL-4 + anti-TNFα mAb
H - anti-IL-4 + anti-TNFα + anti-IL-1β mAb

The levels of eosinophils in the blood of IL-$5^{-/-}$ mice in the presence of s.c. treatment with anti-IL-4, anti-TNF-α, anti-IL-1β or control mAbs, singularly or in combination with each other, were inversely proportional to the levels of eosinophils that accumulated at sites of CLPR. The s.c. treatment of IL-$5^{-/-}$ mice with the control mAbs or anti-IL-1β mAb (in the absence of the anti-IL-4 and or anti-IL-1β mAbs) simultaneously, did not effect eosinophilic responses in the blood in response to Ova. In contrast, the mice that received anti-IL-4 and/ or anti-TNF-α mAbs did not have reduced blood eosinophil levels 6 hours after the s.c. administration of Ova , when compared with the blood eosinophil levels in these mice prior to s.c.administration of Ova (figure VII.2b).

VII.3.3 Eosinophil recruitment at sites of cutaneous late-phase reaction is not significantly inhibited in the absence of IL-4 and/or TNF-α function.

In the absence of TNF-α function [as engendered by the absence of the TNF R1 (P55) and R2 (P75) receptors], the accumulation of eosinophils at sites of CLPR was not affected when measured 6 hours after s.c. Ova provocation (figure VII.3). Similarly, eosinophil levels in the blood prior to, or 6 hours after, s.c. Ova provocation were also not affected in absence of TNF-α function. As previously observed in IL-$5^{-/-}$ and IL-$5^{+/+}$ mice (Chapter V), there was a significant reduction in the levels of eosinophils in the blood of the sensitised P55/P75$^{-/-}$ and P55/P75$^{+/+}$ mice when compared with levels in these mice before s.c. administration of Ova .

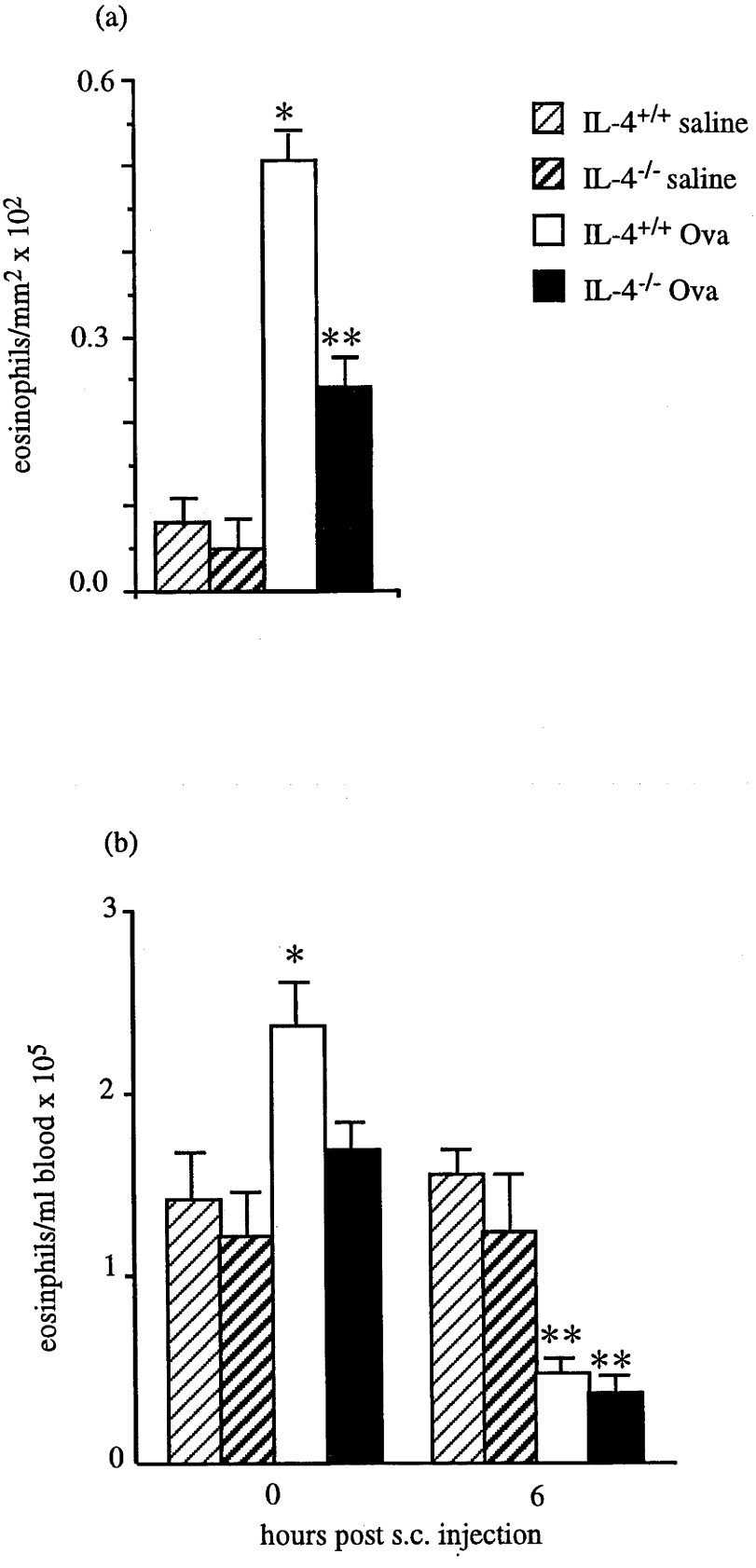
In contrast to P55/P75$^{-/-}$ mice, IL-$4^{-/-}$ mice had impaired eosinophil accumulation at sites of CLPR when compared to their wild type counterparts (IL-$4^{+/+}$)(figure VII.4a). Sensitised IL-$4^{+/+}$ mice, but not sensitised IL-$4^{-/-}$ mice had significantly ($P < 0.05$) higher eosinophil levels in the blood than the non sensitised controls. Furthermore, the blood levels of eosinophils in the sensitised IL-$4^{-/-}$ mice were significantly ($P < 0.05$) lower that those observed in the sensitised wild type mice before s.c. Ova provocation. Since sensitised IL-$4^{-/-}$ mice exhibited reduced circulating eosinophil levels (compared with the sensitised wild type mice) prior to the induction of CLPR, it was hypothesised that the reduced accumulation of eosinophils in these mice during CLPR may reflect a decreased circulating eosinophil pool rather than impaired eosinophil recruitment processes. In order to clarify the mechanism involved in the attenuation of tissue eosinophilia, sensitised and non-sensitised IL-$4^{+/+}$ and IL-$4^{-/-}$ mice were given an i.v. adoptive transfer of 1 x 10^6 H33342 fluorescently labelled eosinophils 4 hours after the s.c. injection of Ova or saline. Two hours later the accumulation of fluorescent eosinophils was quantified at the s.c. site of injection.

Figure VII.3 ***The role of TNF-α in eosinophil trafficking during CLPR in mice.***

P55/P75$^{+/+}$ and P55/P75$^{-/-}$ mice were sensitised to Ova by i.p injection on days 0 and 12 and on day 24 were given a s.c. injection of 50 μg Ova in 100 μl saline with 900 μl of air. Non-sensitised mice received s.c. saline only. Mice were sacrificed 6 hours later and eosinophil numbers at s.c. sites and in the blood quantified. Eosinophilic responses to Ova at sites of CLPR (a) or in the blood (b) were not affected in P55/P75$^{-/-}$ mice when compared to P55/P75$^{+/+}$ mice. Results represent (a) mean eosinophils/mm^2 ± SEM and (b) mean eosinophils/ml of blood ± SEM of groups of 6 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) $*P < 0.01$ when compared with the saline controls. (b) $*P < 0.05$ when compared with the non-sensitised groups and $**P < 0.05$ when compared with the saline controls. No significant difference in mean levels of eosinophil accumulation in the skin (a) or the mean eosinophils levels in the blood (b) were detected between the P55/P75$^{+/+}$ and P55/P75$^{-/-}$ mice groups of the same treatment.

Figure VII.4 ***The role of IL-4 in eosinophil trafficking during cutaneous late-phase reaction.***

IL-$4^{+/+}$ mice and IL-$4^{-/-}$ mice were sensitised to Ova by i.p injection on day 0 and 12 and on day 24 the mice received a s.c. injection of 50 μg Ova in 100 μl saline with 900 μl of air. Non-sensitised groups received s.c. saline and air only. (a) The accumulation of eosinophils at sites of CLPR at 6 hours was reduced in the IL-$4^{-/-}$ mice. Results represent mean eosinophils/mm^2 ± SEM of groups of 5-6 mice. (b) The levels of eosinophils in the blood of the sensitised IL-$4^{-/-}$ mice were significantly lower than those in the sensitised IL-$4^{+/+}$ mice prior to s.c. Ova administration. Eosinophil numbers in the blood 6 hours after the induction of CLPR were similar in IL-$4^{-/-}$ and IL-$4^{+/+}$ mice when compared between the same treatment. Data represents mean eosinophils/ml blood ± SEM of groups of 6 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) $P < 0.05$ compared with saline controls at the same time point and $^{**}P < 0.05$ compared with sensitised IL-$4^{+/+}$ mice at 6 hours post s.c. Ova administration and when compared with the saline controls. (b) $^{*}P < 0.05$ compared with sensitised IL-$4^{+/+}$ mice prior to s.c. Ova administration and $^{**}P < 0.05$ compared with saline controls.


(a)
0.6
0.3
0.0
eosinophils/mm^2 x 10^2
*
**
IL-4$^{+/+}$ saline
IL-4$^{-/-}$ saline
IL-4$^{+/+}$ Ova
IL-4$^{-/-}$ Ova
(b)
3
2
1
0
eosinphils/ml blood x 10^5
*
**
**
0
6
hours post s.c. injection

Following the adoptive transfer of H33342 labelled eosinophils, fluorescent cells were detectable in the blood of the recipients. The levels of fluorescent cells in the circulation were equivalent in all groups of sensitised and non-sensitised IL-$4^{+/+}$and IL-$4^{-/-}$ mice when determined 10 minutes after i.v. adoptive transfer (figure VII.5a). As previously observed in IL-$5^{+/+}$ mice (Chapter V), a significantly ($P < 0.01$) greater number of fluorescent eosinophils accumulated at sites of Ova-induced CLPR in mice than at sites of saline administration (figure VII.5b). Moreover, the ability of fluorescent eosinophils to home to sites of CLPR or s.c. saline administration in mice was not effected in the absence of IL-4 (figure VII.5b).

To determine whether the apparent lack of effect of IL-4 or TNF-α in processes of eosinophil homing at sites of CLPR (as discussed above) was due to the overlapping function between these molecules, sensitised wild type mice were s.c. coadministered with anti-IL-4 and anti-TNF-α mAbs at sites of s.c. Ova administration. In contrast to similar studies (Satoh *et al.*, 1997), the s.c. administration of anti-IL-4 and anti-TNF-α mAb had little effect on the eosinophilic responses in the skin (figure VII.6a) or the circulation (figure VII.6b) when measured at 6 hours after s.c. Ova provocation.

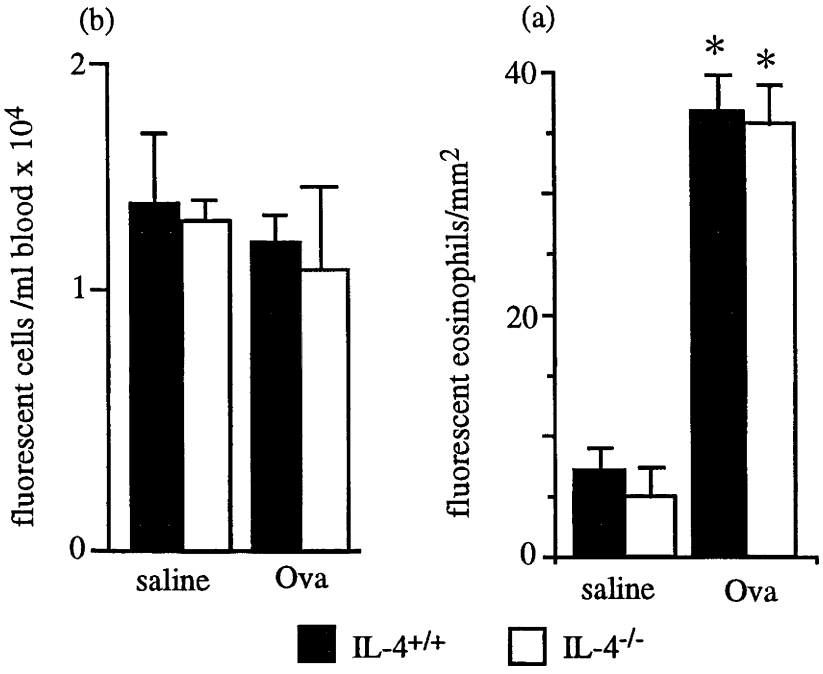


Figure VII.5 ***The role of IL-4 in eosinophil homing to sites of CLPR in mice.***

IL-$4^{+/+}$ mice and IL-$4^{-/-}$ mice were sensitised to Ova by i.p injection on days 0 and 12. On day 24 the mice received a s.c. injection of 50 µg Ova in 100 µl saline with 900 µl of air. Non-sensitised mice received s.c. saline only. Four hours after s.c. injections mice received an i.v. adoptive transfer of 1 x 10^6 H33342-labelled eosinophils. Mice were sacrificed 2 hours later and the accumulation of fluorescent eosinophils/mm^2 on s.c. membrane sections quantified. (a) Adoptively transferred H33342-labelled eosinophils accumulated at sites of CLPR at 6 hours post Ova provocation and the ability of these cells to accumulate was not affected in IL-$4^{-/-}$ mice. Significantly greater levels of H33342-labelled eosinophils accumulated at sites of CLPR than at sites of saline administration. Results represent mean fluorescent cells/mm^2 ± SEM for groups of 5 animals. (b) Fluorescent cells in the blood at 10 minutes post i.v. adoptive transfer were equivalent in all groups of mice. Results represent mean fluorescent cells/ml of blood ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) No significant difference between the means of any group of mice receiving an adoptively transfer of eosinophils were detected. (b) $*P < 0.01$ when compared with saline controls. No significant differences were detected between the means of IL-$4^{+/+}$ and IL-$4^{-/-}$ groups when compared within the same treatment.

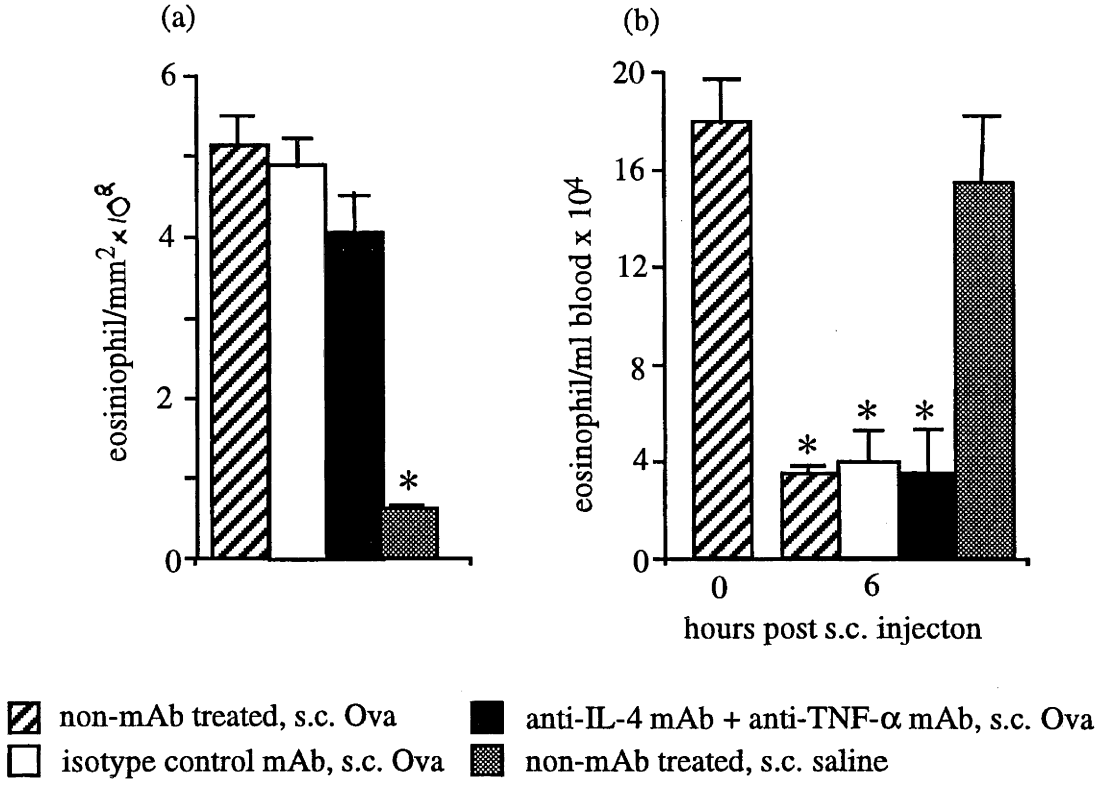


Figure VII.6 ***Treatment of mice with anti-IL-4 and anti-TNF-a mAbs has no effect on eosinophilic responses at sites of CLPR or in the blood of wild type mice.***

IL-$5^{+/+}$ mice (C57BL6) were sensitised to Ova by i.p injection on days 0 and 12. On day 24 mice received a s.c. injection of 900 µls of air with 50 µg of Ova in 100 µl of saline contained either 100 µg of anti-IL-4 mAb and 100 µg of anti-TNF-α mAb or 200 µg of isotype control mAb (GL113). For comparison mice were also given a s.c. injection of Ova in saline with 900 µl of air in the absence of mAb treatment. Mice were sacrificed 6 hours later and the accumulation of eosinophils at sites of CLPR quantified. Eosinophil levels in the blood were also quantified at this time as well as prior to s.c. injection. Data represents (a) mean eosinophils/mm^2 ± SEM and (b) mean eosinophils/ml blood ± SEM of groups of 5 mice. The significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Differences in means were considered significant if $P < 0.05$. (a) $^*P < 0.01$ when compare with all groups that received s.c. Ova. (b) $^*P < 0.01$ compared with levels prior to s.c. injection of Ova. No significant differences were detected between the means of the group treated with anti-IL-4 and anti-TNF-α mAbs, the group treated with the isotype control mAb and the non-antibody treated group at 6 hours post s.c. Ova provocation.

VII.4 DISCUSSION.

Basal eosinophil recruitment into peripheral tissues is impaired in IL-$5^{-/-}$ mice (Chapter V) and the nature of this impairment indicates that adhesion systems are defective in these animals. In contrast, the homing of eosinophil to sites of allergic inflammation is not inhibited in IL-$5^{-/-}$ mice (Chapter V) and thus, inflammatory mediators may restore adhesion systems in IL-5 deficient mice.

Previous observations of mice during allergic airways inflammation (Gonzalo *et al.*, 1996a) indicated that the recruitment of eosinophils at sites of inflammation is dependent on both ICAM-1 and VCAM-1 adhesion pathways. The results in this Chapter also support a role for these two adhesion pathways in eosinophil recruitment at sites of CLPR. The treatment of IL-$5^{+/+}$ mice and IL-$5^{-/-}$ mice with anti-ICAM-1 and anti-VCAM-1 mAbs inhibited the accumulation of eosinophils at sites of CLPR 6 hours after antigen provocation. Thus, not only do these molecules participate in eosinophil recruitment into inflamed tissues, they are also activated independently of IL-5 during allergy. Since the treatment of mice with anti-ICAM-1 and anti-VCAM-1 mAbs was more effective at inhibiting the accumulation of eosinophils at sites of CLPR than either mAb alone, these adhesion molecules may promote eosinophil trafficking in tissues independently of one another. The i.v. treatment of mice with anti-VCAM-1 and anti-ICAM-1 mAbs also inhibited the reduction of eosinophil numbers in the blood that are associated with CLPR. This indicates that the inhibition of the accumulation of eosinophils at sites of allergic inflammation by these mAbs, was coupled to mechanisms regulating the sequestration of eosinophils from the circulation into inflammed tissues.

Eosinophil recruitment at sites of CLPR in IL-$5^{-/-}$ mice was reduced in the presence of anti-IL-4 or anti-TNF-α mAbs, but not in the presence of anti-IL-1β mAb. Thus, although IL-1β stimulates eosinophil adhesion and transmigration at the vascular endothelium *in vitro* (Ebisawa *et al.*, 1992), this cytokine is redundant or not involved in eosinophil recruitment processes during allergy. Interestingly, previous reports indicated that the treatment of cyclophosphamide-treated mice with anti-IL-1β mAb reduced the accumulation of eosinophils at sites of hapten administration (Satoh *et al.*, 1997). Thus, there may be a differential requirement for this cytokine in eosinophil trafficking in various eosinophilia models. Furthermore, the cyclophosphamide model of eosinophilia may not be suitable for the study of eosinophil trafficking during allergy.

The s.c. administration of both anti-IL-4 and anti-TNF-α mAbs to IL-$5^{-/-}$ mice was no more effective at reducing the accumulation of eosinophils at sites of CLPR than either of these mAbs alone. Thus, IL-4 and TNF-α may act together to promote eosinophil recruitment at sites of allergic inflammation. Both IL-4 and TNF-α may promote

eosinophil recruitment by activating adhesion systems that promote eosinophil tethering and transmigration at the vascular endothelium at the site of allergic inflammation (Briscoe *et al.*, 1992, Pober *et al.*, 1996). In light of the recent demonstration that both IL-4 and TNF-α act in synergy to promote the expression of VCAM-1 on the vascular endothelium *in vitro* (Iademarco *et al.*, 1995), it is possible that these cytokines act together to promote eosinophil recruitment at sites of allergic inflammation. Interestingly, in the absence of TNF-α function (as effected by the deletion of the receptors for this cytokine), mice exhibited normal eosinophilic responses at cutaneous sites of allergic inflammation. Thus, in the presence of IL-5, TNF-α may become redundant in processes that promote the accumulation of eosinophils at sites of allergic inflammation.

Although early (6 hours post antigen s.c. administration) eosinophilic responses in IL-$4^{-/-}$ mice were reduced at sites of CLPR, the signal for eosinophil homing at these sites in these mice was not impaired. Circulating eosinophil levels in IL-$4^{-/-}$ mice do not increase during sensitisation to antigen (unlike their wild type counterparts) and thus decreased eosinophil recruitment at sites of CLPR may reflect the reduced circulating eosinophil pool. The inability of IL-$4^{-/-}$ mice to develop blood eosinophilia following sensitisation may involve the reduced capacity of these animals to produce IL-5 (effected by the inability of these mice to produce Th_2 type responses) (Kopf *et al.*, 1993, Gonzalo *et al.*, 1996).

Although a role for IL-4 or TNF-α in eosinophil recruitment to sites of allergic inflammation was demonstrated in IL-$5^{-/-}$ mice, the treatment of wild type mice with both anti-IL-4 and anti-TNF-α mAbs had no significant effect on either the accumulation of eosinophils at sites of inflammation or antigen induced reductions in the circulating eosinophil pool. This indicates that in the presence of IL-5, both IL-4 and TNF-α become redundant in eosinophil recruitment processes at sites of allergic inflammation. These results are of considerable importance since they suggest that there are multiple pathways of eosinophil recruitment at sites of allergic inflammation. Thus, the therapeutic intervention of allergic disease by the specific targeting of individual molecules involved in eosinophil recruitment at sites of inflammation may not be possible. It would be interesting to verify the role of these cytokines in the activation of adhesion pathways at sites of inflammation, particularly mechanisms involving ICAM-1 and VCAM-1. This could be accomplished by the immunohistochemical examination of the expression and activation of adhesion molecule subsets at cutaneous sites of allergic inflammation in IL-$5^{+/+}$ and IL-$5^{-/-}$ mice, in the presence and absence of anti-IL-4 and anti-TNF-α mAbs.

Results in this Chapter suggest of a role for IL-5 in eosinophil recruitment pathways during allergy. Furthermore, these experiments demonstrate that a high degree of

redundancy may exist between inflammatory cytokines that are involved in the promotion of eosinophil recruitment to sites of allergic inflammation, possibly due to the overlapping action of these molecules on adhesion systems. In the absence of IL-5 (but not in it's presence) both IL-4 and TNF-α are required for eosinophil trafficking at sites of CLPR. This suggests that IL-5 has a similar action to IL-4 and TNF-α in the promotion of eosinophil trafficking. TNF-α is not considered to be an eosinophil chemoattractant and in light of the demonstration that eosinophil homing to sites of allergic inflammation is not impaired in IL-$5^{-/-}$ mice (Chapter V), these cytokines may not act as eosinophil chemoattractants at sites of allergic inflammation. As IL-4, TNF-α and IL-5 all induce the expression of adhesion molecules in endothelial cultures, these cytokines probably promote eosinophil adhesion and transmigration at the vascular endothelium, possibly by regulating the expression and activational states of ICAM-1 and VCAM-1.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

VIII.1 INTRODUCTION.

Eosinophil trafficking and activation is a complex process that is potentially regulated by numerous cytokines, chemokines, lipid mediators and small bioactive peptides. Accumulating evidence supports a role for eotaxin and IL-5 as central mediators of selective eosinophil recruitment into tissues, and indicates that both of these cytokines may also regulate eosinophil activation. Recent literature suggests that under basal conditions, eotaxin and IL-5 may act cooperatively to regulate eosinophil homing and tissue accumulation (Collins *et al.,* 1995; Rothenberg *et al.,* 1996). Furthermore, both IL-5 and eotaxin have been shown to regulate the accumulation of eosinophils at sites of allergic inflammation (Foster *et al.*, 1996; Rothenberg *et al.*, 1997). In response to inflammatory stimuli, IL-5 provides the essential signal for the rapid mobilisation of a bone marrow pool of eosinophils and is required in the maintenance of blood eosinophilia (Coffman *et al.*, 1989; Kopf *et al.*, 1996; Foster *et al.,* 1996). In contrast, eotaxin may regulate early-phases of eosinophil recruitment into tissues (Rothenberg et al., 1997). Although previous studies show cooperation between IL-5 and eotaxin in the regulation of eosinophil trafficking (Collins *et al.,* 1995, Rothenberg, *et al.,* 1996), the relationship between these cytokines in eosinophil recruitment and activation was unknown. It was the role of the research presented in this thesis to define the relationship between IL-5 and eotaxin in the regulation of eosinophilic responses in the circulation and in tissues; and to determine if there is a requirement for these cytokines in eosinophil trafficking at basal states and at sites of allergic inflammation. Furthermore, the effect of these two cytokines on eosinophil-induced airways hyperreactivity was also examined.

VIII.2 SUMMARY OF EXPERIMENTAL FINDINGS.

Both IL-5 and eotaxin were found to be potent and selective regulators of eosinophilic responses *in vivo*. When administered systemically, eotaxin and IL-5 potently induced blood eosinophilia. IL-5 mobilised eosinophils from the bone marrow and eotaxin acted independently of this compartment. Systemic eotaxin may recruit eosinophils out of peripheral tissues. Therefore, two mechanism exist by which eosinophils can be rapidly mobilised into the circulation. Eotaxin is produced by tissues and IL-5 from T-cells following antigen provocation. Thus, eotaxin may augment the IL-5 signal by supplementing blood eosinophilia in the early phases of the inflammatory response as well as eliciting a selective chemoattractant signal for eosinophil polarisation and migration (Jose *et al.,* 1994a; Rothenberg *et al.,* 1996).

When administered to the skin, eotaxin and IL-5 potently induced tissue eosinophilia. The effect of eotaxin was highly dose dependent indicating that the action of this molecule is tightly regulated. Furthermore, these cytokines acted in synergy. IL-5 also

synergistically promoted the effects of RANTES and MIP-1α on the accumulation of eosinophils in tissues, but not with lipid mediators (PAF and LTB_4) or ECF-a (Val-Gly-Ser-Glu). Thus, not only is IL-5 a potent eosinophil chemoattractant in its own right, it also synergistically promotes eosinophil chemotaxis in response to CC-chemokines at concentrations that are suboptimal for these cytokines in isolation. Eotaxin, MIP-1α and RANTES may all signal through the chemokine receptor CCR3 and IL-5 could potentiate this pathway. RANTES and MIP-1α also signal through other CCRs which may account for their action on a variety of other cell types. Thus, the unique relationship between IL-5 and CCR3 operation provides a fundamental mechanism for the amplification of signals selectively regulating eosinophil trafficking.

Eotaxin and IL-5 were also found to be potent and selective stimuli of the accumulation of eosinophils in the lungs of mice. Furthermore, these two cytokines acted in synergy. This indicates that eotaxin and IL-5 may promote eosinophil trafficking in both mucosal (airways) and non-mucosal (skin) tissues as well as in the circulation. Although both eotaxin and IL-5 activate eosinophils *in vitro* (Kita *et al.*, 1992, Elsner *et al.*, 1996), these cytokines in combination, did not induce eosinophils to release MBP in the airways. Furthermore, these cytokines alone or in combination did not promote airways hyperreactivity. The additional antigenic stimulus provided by repeated aeroallergen exposure was required to induce the release MBP from eosinophils and for airways hyperreactivity to develop. Notably, the signals derrived from antigen stimulation could not induce airways hyperreactivity in the absence of airways eosinophilia. Interestingly, no damage to the airways was observed in those mice that developed airways hyperreactivity. Thus, airways damage does not necessarily precede the development of airways hyperreactivity. Furthermore, the repeated exposure of the mice to aeroallergen did not affect the levels of eosinophils in the airways. These results indicate that not only were the levels of eosinophils in the airways (in the absence of aeroallergen exposure) sufficient to induced airways hyperreactivity, but the activation of these cells is not solely effected by IL-5 and eotaxin. Interestingly, genetically manipulated mice that over produce IL-5 specifically by airway epithelial cells, develop both airways eosinophilia and hyperreactivity (Lee *et al.*, 1997). ~~Thus, prolonged exposure of airways eosinophils to high levels of IL-5 may induce degranulation leading to airways dysfunction. Furthermore, as indicated by the disease-like pathology in the lungs, non-specific stimuli may also induce eosinophil degranulation in these IL-5 transgenic mice.~~ However, No extracellular MBP was detected in the lungs of these mice. Perhaps IL-5 stimulated the selective release of other cationic proteins from eosinophils or activated eosinophils to release oxygen radicals.

IL-5 may not only potentiate eotaxin-induced eosinophil chemotaxis, but also provide an essential signal for basal eosinophil migration into tissues in response to eotaxin stimuli. The impaired nature of eotaxin signalling in the tissues of $IL\text{-}5^{-/-}$ mice indicates that basal levels of IL-5 control eosinophil transmigration events at the vascular endothelium and suggest a requirement for this cytokine in adhesion molecule function. These studies

further emphasise the broad action of IL-5 on eosinophil function. Interestingly, eotaxin signalling in the blood occurs in the absence of IL-5. Since systemic eotaxin most probably draws eosinophils out of peripheral tissues and not from the bone marrow, eosinophil trafficking within tissues and the migration of this cell out of tissues in response to eotaxin does not require IL-5, in contrast to eosinophil migration into tissues. This suggests that there is a differential usage of adhesion processes between active migration in and transient margination mechanisms in tissues.

The demonstration that systemic eotaxin induces blood eosinophilia in the absence of IL-5 is novel and demonstrates the existence of a pool of eosinophils that can be mobilised independently of IL-5. However, although systemic eotaxin promoted blood eosinophilia it did not enhance eosinophilic responses in tissues, in contrast to IL-5. The systemic coadministration of IL-5 and eotaxin amplified eosinophilic responses in tissues to a greater extent than either of these cytokines alone. Thus, in addition to promoting blood eosinophilia, IL-5 may be required to activate (ie. prime) the eosinophils in the circulation, promoting increased responsiveness to chemotactic stimuli in tissues.

Although eotaxin or IL-5 -induced eosinophil homing in tissues at base line was impaired in IL-$5^{-/-}$ mice, eosinophil trafficking in tissues at sites of allergic inflammation was not. This indicates that inflammatory mediators may overcome any impairment in eosinophil homing that occur in the absence of IL-5 at basal states. IL-4 and TNF-α may contribute to this process since in the absence of IL-5 these molecules regulate the accumulation of eosinophils at sites of CLPR. Interestingly, IL-5 may also promote eosinophil trafficking at sites of CLPR since both IL-4 and TNF-α become redundant in this process in the presence of IL-5. These results indicate that there may be a high degree of redundancy between these cytokines in the events regulating eosinophil trafficking to sites of allergic inflammation. All of these molecules regulate the expression of adhesion molecules, and thus, the redundancy between these cytokines may reflect their overlapping activation of adhesion pathways at sites of allergic inflammation.

The development of airways eosinophilia and airways dysfunction during allergic airways inflammation was severely attenuated in IL-$5^{-/-}$ mice (Foster *et al.*, 1996). However, eosinophil homing to the inflamed lungs is not impaired in IL-$5^{-/-}$ mice (Chapter V). Thus, the attenuation of the accumulation of eosinophils in the lungs IL-$5^{-/-}$ mice may reflect their inability to develop blood eosinophilia in response to antigenic stimuli. Not only was eosinophil recruitment at sites of antigen provocation independent of IL-5, this cytokine was not required for the development of airways hyperreactivity. Sensitised IL-$5^{-/-}$ mice developed increased airways responsiveness to the spasmogen, β-methylcholine, following multiple exposures to aeroallergen. Furthermore, as in wild type mice, the degree of airways hyperreactivity correlated with the levels of eosinophils

and the level of MBP in the airways. These results suggest that eosinophils and their products directly mediate airways hyperreactivity. Further studies showed that by amplifying circulating eosinophil levels in IL-5$^{-/-}$ mice by adoptive transfer of eosinophils or by the systemic administration of eotaxin, both airways eosinophilia and airways hyperreactivity are enhanced. Interestingly, the adoptive transfer of eosinophils that had prior exposure to IL-5, increased airways reactivity to a greater level than the systemic administration of eotaxin; even though both treatments induced equivalent and specific increases in the levels of eosinophils in the airways. Thus, although IL-5 is not required for the induction of airways hyperreactivity following aeroallergen provocation, it does amplify it. This may involve the ability of this cytokine to prime eosinophils, inducing heightened sensitivity to degranulatory stimuli. The specific localisation of eosinophils in regions near the airways smooth muscle, but not in regions near the airways epithelium, suggests that eosinophils induce airways hyperreactivity by effecting airways smooth muscle function rather than through airways epithelial lining disruption. Furthermore, as previously observed, airways hyperreactivity occurred in the visible absence of airways damage, (including airway epithelial shedding) and thus, it is concluded that airways hyperreactivity precedes airways damage. The primary difference between studies by Foster et al., (1996) and those here is the amount and number of exposure of IL-5$^{-/-}$ mice to aeroallergen. In investigations in this thesis, IL-5$^{-/-}$ mice received an extra day of Ova Inhalation and the dose was 150 mg of Ova per 30 minute aerosol period [as opposed to 100 mg per 30 minute aerosol period used by Foster et al., (1996)]. Thus, the antigen load to the airways regulates the number of eosinophils recruited to the lung and the subsequent impact of the eosinophil regulates airways hyperreactivity. In unpublished observations, IL-5$^{-/-}$ mice that are chronically exposed to Ova develop pronounced foci of airways eosinophils and airways hyperreactivity without developing a blood eosinophilia. Thus, in chronic models of allergic airways disease it would appear that pathophysiology can occur independently of IL-5.

In contrast to IL-5, eotaxin may direct eosinophil chemotaxis at cutaneous sites of allergic inflammation. Elevated levels of eotaxin mRNA were observed at skin and lung sites of allergic inflammation soon after antigen provocation and were dependent on IFNγ. Interestingly, the expression of eotaxin in the lungs, but not in the skin at sites of allergic inflammation was also dependent on IL-4. Thus, the expression of this chemokine may be differentially regulated at non-mucosal and mucosal sites of allergic inflammation.

Eotaxin may not only regulate eosinophil trafficking at base line and during allergy, but may also regulate eosinophilic responses to non-allergic stimuli. Eotaxin deficient mice developed abnormally high levels of eosinophils at s.c. sites of saline administration. Eotaxin may also regulate the responsiveness of eosinophils to itself, as eosinophilia in the blood following the systemic administration of eotaxin was amplified in eotaxin$^{-/-}$

mice. This may reflect a down regulation of eotaxin signalling through CCR3, that has previously been observed *in vitro* (Rothenberg *et al.*, 1996). Although eotaxin does not appear to regulate IL-5 induced blood eosinophilia, it may participate in IL-5-induced eosinophil differentiation. In the absence of eotaxin, bone marrow eosinophil levels rise rapidly following the systemic administration of IL-5, and cultures of bone marrow cells from eotaxin$^{-/-}$ mice rapidly differentiate into eosinophils following the addition of IL-5. However, eotaxin is not significantly involved in eosinophil differentiation at resting states, as eotaxin deficient mice have normal levels of eosinophils in bone marrow. Eotaxin may also regulate eosinophil mobilisation from the bone marrow following antigen provocation, as bone marrow eosinophil mobilisation was heightened in eotaxin deficient mice during cutaneous allergy and this phenomena may involve an eosinophil hypersensitivity to IL-5.

VIII.3 THE PROPOSED ROLES OF IL-5 AND EOTAXIN IN EOSINOPHIL TRAFFICKING.

The results presented in this thesis demonstrates that both IL-5 and eotaxin are important regulators of eosinophil trafficking at basal states and during allergy. The following is a proposal of the suspected roles of IL-5 and eotaxin in these processes.

At resting states eosinophil differentiation may be regulated by a range of cytokines, including eotaxin and IL-5. IL-5 promotes eosinophil differentiation and eotaxin may regulate this process. However, these two cytokines do not play obligatory roles in eosinophil differentiation, as eosinophil differentiation occurs in their absence. In the circulation, eosinophil levels may be regulated by both IL-5 and eotaxin. The basal levels of expression of eotaxin in numerous tissues throughout the body (Rothenberg *et al.*, 1995a, 1995b), suggests that eotaxin may also regulate resting state eosinophil recruitment through peripheral tissues. Basal levels of IL-5 may assist in this process by maintaining adhesion molecule function at the vascular endothelium. Furthermore, IL-5 may act in synergy with eotaxin (and other C-C chemokines?) to promote eosinophil trafficking in tissues. This would allow each of these cytokines to effectively signal at concentrations that are suboptimal for the molecules individually. Eosinophil trafficking at resting state is summarised in figure VIII.1.

The selective trafficking of eosinophils at sites of allergic inflammation may also be regulated by eotaxin and IL-5. Following antigen provocation, eotaxin is produced at sites of inflammation. Increased systemic levels of this cytokine may induce the migration of eosinophils out of non-inflamed tissues into the circulation. IL-5 produced at sites of inflammation (probably after eotaxin) also amplifies the circulating eosinophil pool, by mobilising eosinophils from the bone morrow into the circulation. Over longer

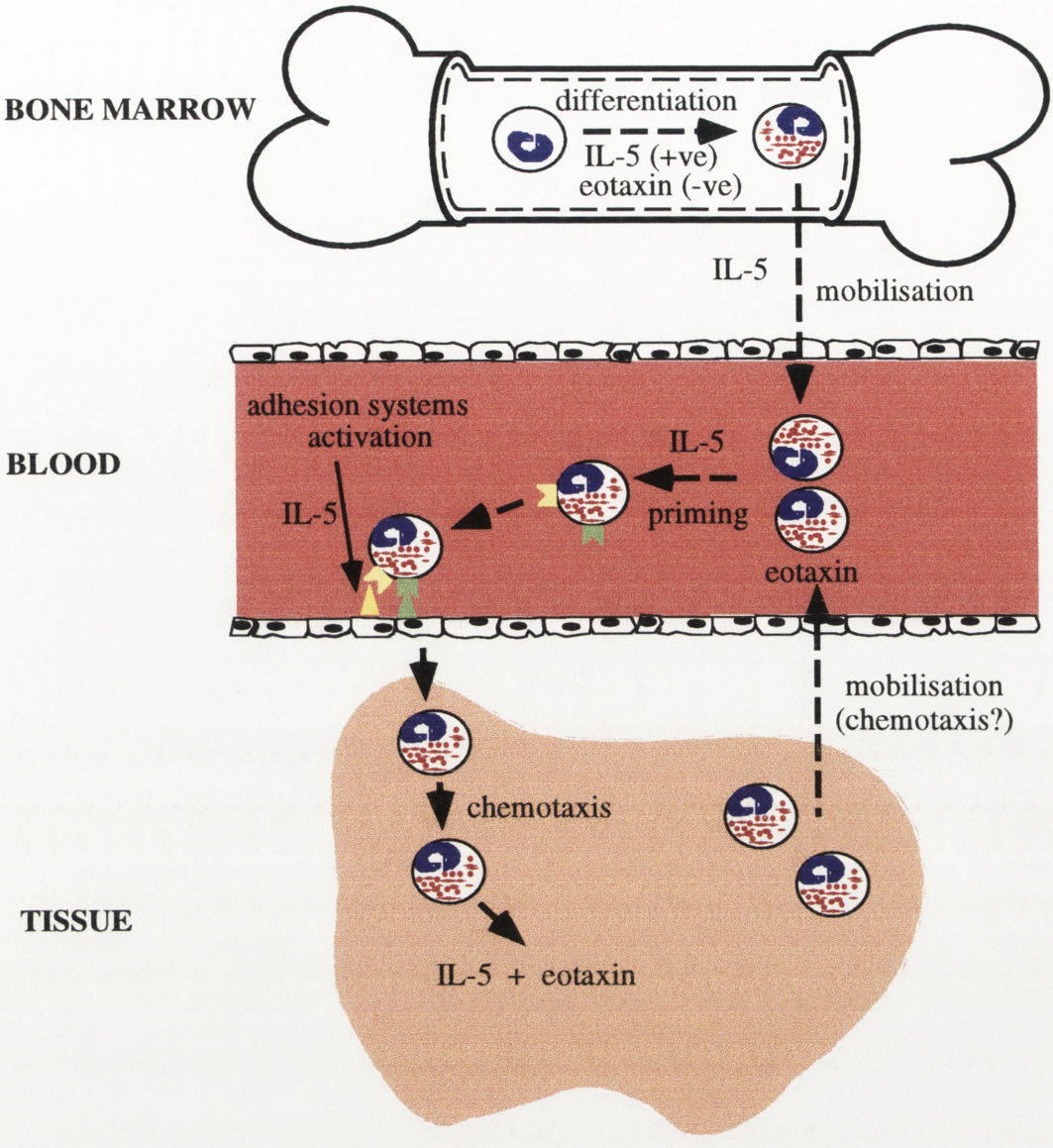


Figure VIII.1 ***The role of IL-5 and eotaxin in eosinophil trafficking at basal states.***

IL-5 differentiates precursor cells into eosinophils in the bone marrow and eotaxin may regulate this process. Systemic IL-5 mobilises eosinophils into the circulation and primes them, leading to enhanced chemotactic responsiveness. In addition IL-5 may also activate adhesion molecule expression on the eosinophil and regulate the function of adhesion systems at the vascular endothelium. Eotaxin may also regulate eosinophil levels in the circulation by recruiting eosinophils out of tissues. In addition, IL-5 and eotaxin, may also regulate eosinophil trafficking in tissues as part of immune surveillance. Furthermore, in this capacity IL-5 may promote effective and selective eosinophil migration by enhancing the effects of CC-chemokines.

periods of time, systemic IL-5 may also promote the differentiation of eosinophils in the bone marrow for the long term development and maintenance of blood eosinophilia. Furthermore, eotaxin may negatively regulate this process.

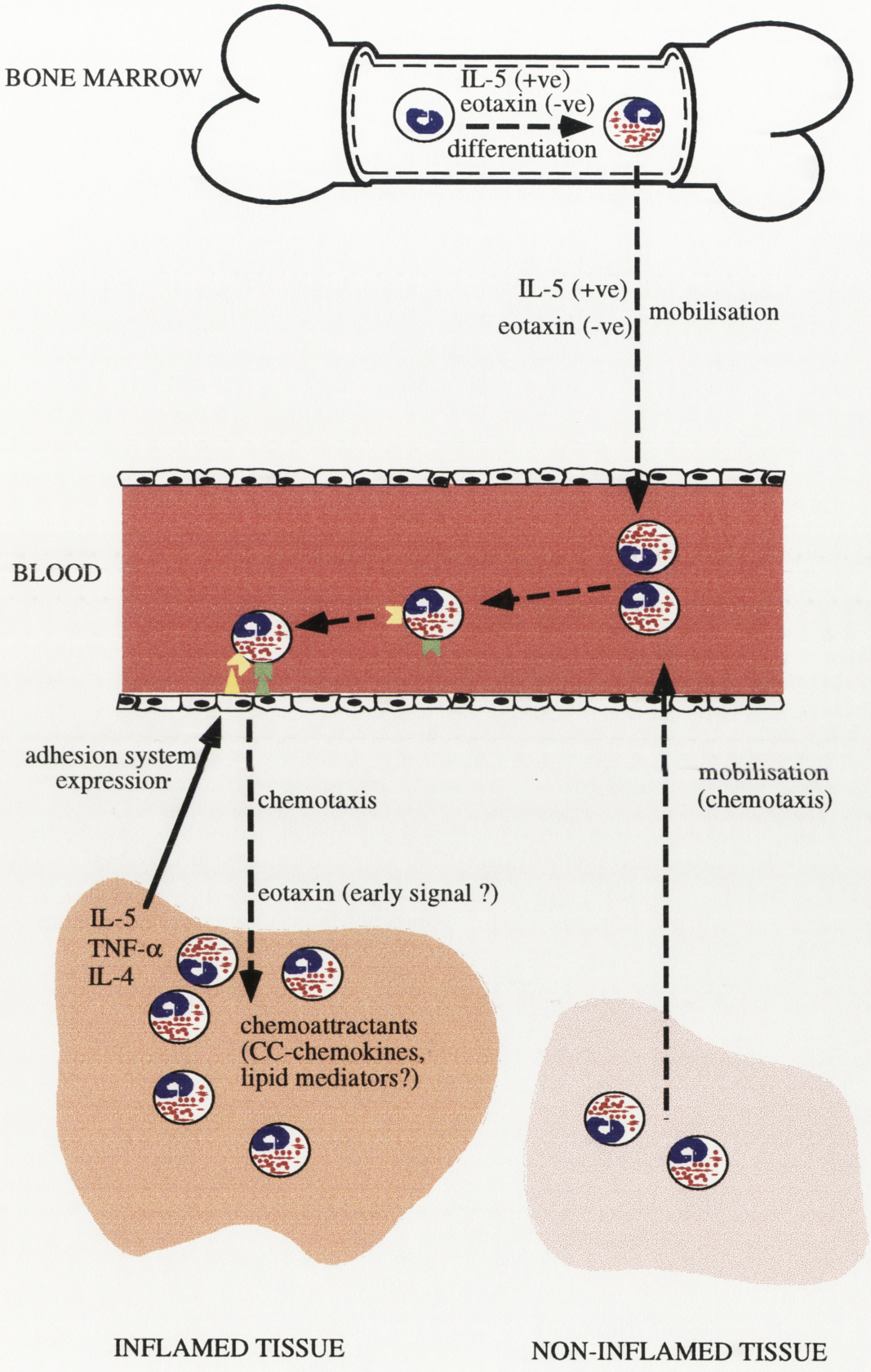
IL-5, in addition to IL-4 and TNF-α may promote eosinophil transmigration at the vascular endothelium in inflamed tissues by inducing the expression of complementary adhesion molecules. Eosinophil polarisation and mobilisation in tissues is promoted by chemotactic gradients formed by the release of chemoattractants at the loci of inflammation. Eotaxin may promote eosinophil chemotaxis during the early stages of the inflammatory response, becoming redundant during the later stages of the inflammatory reaction due to the production of other eosinophil chemoattractants, such as RANTES and MIP-1α (Alam *et al.*, 1996; Humbert *et al.*, 1997). Interestingly, although IL-5 is chemotactic for eosinophils *in vivo*, this cytokine does not appear to be involved in eosinophil homing at sites of inflammation during allergy.

In the airways, eosinophils preferentially localise in regions near the airways smooth muscle in response to IL-5 and eotaxin expression by recombinant vaccinia viruses and following aeroallergen provocation. Inflammatory mediators may then activate eosinophils to degranulate and release MBP. IL-5 at sites of inflammation may not only prime eosinophils to respond to degranulatory stimuli, but at high concentrations and over long periods of time may itself promote eosinophil degranulation (Lee *et al.*, 1997). MBP causes airways dysfunction by allosteric blocking of inhibitory pathways of smooth muscle contraction, leading to hypersensitivity to spasmogenic stimuli (Gleich *et al.*, 1995). Over prolonged periods eosinophils may also induce damage to the airways by amplifying the inflammatory response through the release of cytotoxic molecules, including cationic proteins and lipid mediators, in addition to the generation of highly reactive oxygen species (Bruijnzeel, 1989; Gleich, 1990; Gleich *et al.*, 1994). The role of IL-5 and eotaxin in the regulation of eosinophil trafficking and activation at sites of allergic inflammation are summarised in figure VIII.2.

Interestingly, although IL-5 and eotaxin potently induce the recruitment of eosinophils into tissues; these molecules are not obligatory for eosinophil homing at sites of allergic inflammation. In the absence of eotaxin eosinophil homing is diminished however, indicating that this chemokine promotes eosinophil chemotaxis at sites of allergen provocation. During allergy, IL-5 appears to primarily promote eosinophil differentiation and the development of blood eosinophilia, as well as prime eosinophils for enhanced responsiveness to chemotactic and degranulatory stimuli. Thus, the inhibition of IL-5 as a therapy for allergic disease such as asthma may be effective, since it would reduce the available eosinophil pool. Eotaxin acts in synergy with IL-5 and plays a fundamental role

Figure VIII.2 ***The role of IL-5 and eotaxin in the regulation of eosinophil trafficking at sites of allergic inflammation.***

The secretion of IL-5 and eotaxin at sites of allergic inflammation elevates systemic levels of these cytokines. IL-5 induces mobilisation of bone marrow eosinophils into the circulation and eotaxin may sequester eosinophils out of non-inflamed tissues. IL-5 also induces eosinophil differentiation in the bone marrow. Eotaxin may regulate IL-5-induced eosinophil differentiation and the mobilisation from bone marrow. IL-5 in the circulation primes eosinophils for enhanced chemotactic responsiveness, and induces the expression of adhesion molecules on these cells. At sites of inflammation, the overlapping actions of IL-5, IL-4 and TNF-α may promote complementary adhesion molecule expression at the vascular endothelium and promote eosinophil adhesion/transmigration. Chemoattractant gradients generated at sites of inflammation induce cell polarisation and migration towards the inflammation site. Eotaxin may promote eosinophil chemotaxis during the early stages of inflammation.


BONE MARROW
IL-5 (+ve)
eotaxin (-ve)
differentiation
IL-5 (+ve)
eotaxin (-ve)
mobilisation
BLOOD
adhesion system
expression
chemotaxis
mobilisation
(chemotaxis)
eotaxin (early signal ?)
IL-5
TNF-α
IL-4
chemoattractants
(CC-chemokines,
lipid mediators?)
INFLAMED TISSUE
NON-INFLAMED TISSUE

in regulating eosinophil chemotaxis. Targetting the action of both molecules would provide a powerful therapeutic approach to downregulating tissue eosinophilia.

In conclusion, IL-5 and eotaxin play fundamental roles in the regulation of eosinophil function. Notably both cytokines can regulate blood and tissues eosinophilia in response to allergic stimuli. In particular, the migration of eosinophils is regulated by a unique signalling arrangement between IL-5 and chemokines that ~~operate~~ activate CCR3 which provides a mechanism that synergistically and selectively potentiates eosinophilia. Modulation of IL-5 and eotaxin signalling may provide a significant advance in the treatment of eosinophil derived pathophysiology in allergic disease.

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